Extracellular iron diminishes anticancer effects of vitamin C: An in vitro study

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In vitro studies have shown that hydrogen peroxide (H₂O₂) produced by high-concentration ascorbate and cell culture medium iron efficiently kills cancer cells. This provided the rationale for clinical trials of high-dose intravenous ascorbate-based treatment for cancer. A drawback in all the in vitro studies was their failure to take into account the in vivo concentration of iron to supplement cell culture media which are characterized by low iron content. Here we showed, using two prostate cancer cell lines (LNCaP and PC-3) and primary astrocytes, that the anticancer/cytotoxic effects of ascorbate are completely abolished by iron at physiological concentrations in cell culture medium and human plasma. A detailed examination of mechanisms showed that iron at physiological concentrations promotes both production and decomposition of H₂O₂. The latter is mediated by Fenton reaction and prevents H₂O₂ accumulation. The hydroxyl radical, which is produced in the Fenton reaction, is buffered by extracellular proteins, and could not affect intracellular targets like H₂O₂. These findings show that anticancer effects of ascorbate have been significantly overestimated in previous in vitro studies, and that common cell culture media might be unsuitable for redox research.

A number of finished (phase I) and still ongoing clinical trials have examined the possible benefits of intravenous ascorbate (Asc) therapy in cancer treatment. High pharmacological concentrations of Asc in the blood (approximately 5–10 mM range) are achievable only by intravenous application and appear to be safe and tolerated⁴–⁷. The key pre-clinical rationale for such trials is found in consistent results from more than 20 in vitro studies which concluded that pharmacological Asc efficiently kills over 50 cancer cell lines, leaving most of the examined primary (normal) cell types unaffected⁴,⁵. Furthermore, the mechanisms of Asc’s anticancer activity were described by Chen and co-workers⁶, and later confirmed by others⁷,⁸, showing that Asc is a pro-drug for extracellular generation of hydrogen peroxide (H₂O₂) and that iron is essential for H₂O₂ production. In brief, Asc reduces iron (Fe³⁺ to Fe²⁺), which further reacts with molecular oxygen forming the superoxide radical anion. Superoxide undergoes (non)enzymatic dismutation to produce H₂O₂. Supra-physiological concentrations of H₂O₂ enter the cell and exert cytotoxic effects. Both apoptotic and necrotic pathways of cell death were implicated, with necrosis more prevalent at increasing Asc doses⁴.

A careful inspection of all the available reports regarding the anticancer effects of Asc reveals a systematic omission. Namely, there is not a single study of the four-component system (Asc, iron, molecular oxygen and cancer cell lines) that has examined the effects of different concentrations of iron. All the in vitro studies in question have been conducted using commercial cell culture media, usually RPMI-1640 or DMEM supplemented with 10% (v/v) foetal calf serum (FCS). While DMEM contains only 0.25 µM ferric nitrate (Sigma-Aldrich formulation), iron is not a regular component of RPMI-1640 but probably exists via impurities. Concentrations of iron in FCS are not consistent and vary between manufacturers and batches [usually 2–5 µM in 10% (v/v) FCS]. In one particular study, the concentration of iron in 10% (v/v) FCS was 4.2 ± 1.1 µM, while another group found ~3 µM Fe in RPMI-1640 with 10% (v/v) FCS⁹. One peculiar detail is that iron chelators have been used in some studies in order to examine the effects of sequestration of (an unknown amount of) iron on Asc cytotoxicity⁴. The key drawback in all the studies is that the concentration of iron was not altered, measured or taken into consideration whatsoever.

The fundamental problem behind the drawback lays in the fact that physiological milieus of interest (human plasma and interstitial fluid) contain much more iron compared to cell culture media. Total concentration of iron in plasma is in the range of 10–30 µM¹¹, and even higher in cancer patients¹²,¹³, as well as in patients receiving
Figure 1 | Effects of pharmacological ascorbate and physiological iron on the viability of cancer and primary cells according to MTT and CV assays. (a) LNCaP and (b) PC-3 cells treated in cell culture medium (RPMI-1640 + 10% FCS) with different concentrations of supplemented iron. * - statistically significant (p < 0.05) compared to control with the same amount of supplemented iron. (c) Primary astrocytes treated with Asc (5 mM) and/or iron (30 µM) in cell culture medium. (d) LNCaP (left) and PC-3 (right) in human plasma; Bars not sharing a common letter are significantly different (p < 0.05). (e) LNCaP and (f) PC-3 cells treated in cell culture medium with different % (v/v) of FCS; Bars not sharing a common letter are significantly different (p < 0.05); * - non-significant compared to control with the same % (v/v) of FCS. Statistical analysis was performed using one-way or two-way ANOVA with post hoc Duncan test.

**Results**

Total concentration of iron in the cell culture medium applied to maintain cells (RPMI-1640 + 10% FCS) was 5.6 ± 1.3 µM. This is considerably lower compared to the iron concentration in plasma (18.4 ± 5.9 µM; p < 0.001). The level of iron in the plasma of six volunteers ranged between 12–28 µM (Supplementary Table S1). Incubation with pharmacological Asc (5 and 10 mM) in cell culture medium resulted in a drastic decrease (~80%) in the number of viable LNCaP and PC-3 cells (Fig. 1a, b). The cell culture medium was then supplemented with iron in the form of ferric ammonium citrate (FAC), in order to mimic the level of iron in plasma/interstitial fluid. The concentrations of supplemented iron were 5, 10, 15, and 30 µM, resulting in the total iron concentrations of approximately 10, 15, 20, and 35 µM (~5 µM already found in the medium + supplemented iron). The addition of iron at a concentration as low as 5 µM (resulting in a lower physiological limit for iron level), annihilated the cytotoxic effects of Asc. All other iron concentrations inhibited the cytotoxic effects of Asc as well. Additional experiments using FeCl₃ were performed in order to eliminate the possibility that the observed effects are mediated by FAC and not by iron per se. Ferric chloride completely inhibited the cytotoxic effects of Asc (data not shown). It should be pointed out that iron at physiological concentrations provided protection irrespective of metastatic potential.
radical (marker of H$_2$O$_2$ production) in cell culture medium samples that were collected after incubation of LNCaP or PC-3 cells for the purposes of viability measurements (results presented in Fig. 1); Box: Characteristic EPR signal of ascorbyl radical with spectral simulation (pale); a rapid and transient O$_2$ production from H$_2$O$_2$ breakdown (Fig. 2b).

molecules for each O$_2$ produced (2H$_2$O$_2$ → O$_2$ + 2H$_2$O). Of note, no CAT activity could be detected in the plasma samples. Of note, no CAT activity could be detected in the plasma samples; Bars not sharing a common letter are significantly different (p < 0.05). (d) The concentration of molecular oxygen and the rate of O$_2$ consumption in plasma supplemented with Asc (5 mM). Characteristic polarographic recordings obtained on plasma samples from three healthy volunteers (total Fe concentrations: volunteer (1) 23.0 µM; (2) 15.4 µM; (3) 24.2 µM) are presented. CAT was added at 15 min. Statistical analysis was performed using one-way ANOVA with post hoc Duncan test.

Figure 2 | The effects of ascorbate and iron on hydrogen peroxide production in cell culture medium and human plasma. (a) Concentration of ascorbyl radical (marker of H$_2$O$_2$ production) in cell culture medium samples that were collected after incubation of LNCaP or PC-3 cells for the purposes of viability measurements (results presented in Fig. 1); Box: Characteristic EPR signal of ascorbyl radical with spectral simulation (pale); * - statistically significant (p < 0.05) compared to control (no Fe supplemented). (b) The concentration of molecular oxygen and the rate of O$_2$ consumption in cell culture medium supplemented with Asc (5 mM) or Fe (5 µM) + Asc (5 mM). CAT (600 U) was added at 15 min. The change in O$_2$ concentration following CAT supplementation is presented as mean ± S.D. (c) Concentration of ascorbyl radical in human plasma samples collected after the incubation of LNCaP or PC-3 cells for the purposes of viability measurements; A low level of ascorbyl radical was found in some untreated plasma samples; Bars not sharing a common letter are significantly different (p < 0.05). (d) The concentration of molecular oxygen and the rate of O$_2$ consumption in plasma supplemented with Asc (5 mM). Characteristic polarographic recordings obtained on plasma samples from three healthy volunteers (total Fe concentrations: volunteer (1) 23.0 µM; (2) 15.4 µM; (3) 24.2 µM) are presented. CAT was added at 15 min. Statistical analysis was performed using one-way ANOVA with post hoc Duncan test.

of the two cell lines. A similar experiment was performed on primary astrocytes. Fig. 1c shows that the supplementation of iron also alleviated the cytotoxic effects of Asc on non-cancer cells. Finally, Asc had very little effect on cancer cell viability in human plasma (Fig. 1d). Only a slight decrease in viability was found for LNCaP cells exposed to 10 mM Asc. Supplementary Table S1 shows the results of viability tests for each of 6 plasma samples. Asc (10 mM) provoked a slight decrease of viability of LNCaP cells only in some plasma samples. Of note, no CAT activity could be detected in the plasma using two different assays. This is in line with previous notions that plasma contains no (or very little) CAT.20,21. Further, we modified the level of iron in cell culture medium by increasing the volume percent concentration of FCS, where 10% (v/v) increase add ~5 µM to the total iron concentration. It can be observed that cytotoxic effects of Asc were gradually alleviated with increasing FCS percentage (Fig. 1e, f).

A set of experiments was performed in order to elucidate the mechanisms of iron’s cytoprotective activity. Increased ascorbyl radical production (Fig. 2a) and O$_2$ consumption (Fig. 2b) imply that the generation of H$_2$O$_2$ is higher in the presence of iron at physiological concentration compared to cell culture medium iron. Ascorbyl radical is a by-product of Asc-mediated reduction of iron (Fe$^{3+}$ to Fe$^{2+}$), and represents a marker of H$_2$O$_2$ production in this system. The level of H$_2$O$_2$ was estimated by the addition of CAT which resulted in a rapid and transient O$_2$ production from H$_2$O$_2$ breakdown (Fig. 2b). The amount of O$_2$ produced and the rate of O$_2$ production were much smaller/lowe in the presence of iron at physiological concentration, compared to the system with cell culture medium concentration of iron. If we take into account that CAT uses two H$_2$O$_2$ molecules for each O$_2$ produced (2H$_2$O$_2$ → O$_2$ + 2H$_2$O + O$_2$), the steady-state concentration of H$_2$O$_2$ in the presence of cell culture medium iron is estimated to be around 25 µM. Physiological iron reduced the level of H$_2$O$_2$ to 1.5 µM. This is in contrast to the amount of O$_2$ that was consumed in these systems prior to CAT addition. Further, it can be observed that CAT almost fully restored the level of O$_2$ in the system with cell culture medium concentration of iron. This is not true for physiological iron, implying the production of end-product(s) other than H$_2$O$_2$. Ascorbyl radical is produced in the plasma supplemented with Asc (5 mM). CAT (600 U) was added at 15 min. Statistical analysis was performed using one-way ANOVA with post hoc Duncan test.

Fig. 2a shows that the incubation of cancer cells with Asc in cell culture medium results in a significant decrease in the level of intracellular thiol (-SH) groups, which are the main targets for H$_2$O$_2$-provoked oxidation.24 This is in line with previous findings.25,26 However, in the presence of iron at physiological concentration, the Asc-mediated decrease in the -SH groups was abolished, i.e. iron prevented intracellular oxidation. Fenton reaction (Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + OH$^-$ + 'OH) appears as a plausible mechanism of removal of extracellular H$_2$O$_2$ in the system with physiological iron. Asc is known to propel the Fenton reaction by maintaining iron in Fe$^{2+}$ form, which is otherwise unstable under physiological pH.26,27 We applied biochemical assays in order to quantify extracellular markers of hydroxyl radical (‘OH)-mediated oxidation of proteins and lipids. Fig. 3b shows that the level of oxidation of extracellular proteins was about three times higher in the presence of physiological level of iron compared to culture medium iron concentration. The
iron, as well as iron supplemented without Asc were incapable of protecting cancer cells. However, pharmacological Asc in combination with physiological Fe promoted degradation of H$_2$O$_2$ and diminished its cytotoxic effects.

Two additional mechanisms that might be relevant for Fe-mediated cytoprotection against Asc were examined. It has been speculated that prolonged exposure of cancer cells to Asc might exert cytotoxic effects via provoking iron deficiency$^{28,29}$. However, Fig. 4 shows that the total intracellular concentration of iron remains unaffected in the presence of extracellular Asc, implying that the cytoprotective effects of iron against Asc-provoked cell death are not related to iron deficiency. Further, it is known that the level of hypoxia-inducible factor-1 (HIF-1), a transcription factor that is involved in the regulation of different aspects of cancer cell biology, can be affected by Asc$^{30–32}$, iron$^{33,34}$, and H$_2$O$_2$$^{35}$. Fig. 5 shows characteristic micrographs, intensities and histograms of HIF-1α immunofluorescence. In LNCaP cells, Asc provoked an increase in HIF-1α level. In addition, it can be observed that percentage distribution of Asc-treated LNCaP cells was shifted towards higher fluorescence intensities. Asc-provoked increase in HIF-1α level was diminished by iron (Fig. 5a). PC-3 cells showed a higher constitutive level of HIF-1α. The level was not affected by Asc, but the combination of pharmacological Asc and physiological Fe provoked its decrease (Fig. 5b).

Fig. 6 shows the effects of Asc and Asc + Fe on multi-cellular tumour spheroid culture of LNCaP. It can be observed that Asc at both concentrations applied here exerted only weak tumour static effects, which appear not to be affected by iron supplementation.

Discussion
This is the first study of the anticancer effects of Asc that has taken into account physiological concentrations of iron. Schemes depicted in Fig. 7 summarise what is known about the pro-oxidative anticancer activity of Asc in the cell culture medium, and what we have shown herein to take place in settings that resemble in vivo conditions. Sub-physiological concentrations of iron in cell culture media allow the accumulation of H$_2$O$_2$, which easily crosses the membrane in order to provoke intracellular oxidative damage resulting in cell death (Fig. 7a). On the other hand, when iron is present at the physiological level (even at the lower physiological limit), the decomposition of H$_2$O$_2$ compensates for H$_2$O$_2$ generation and prevents its accumulation (Fig. 7b). The removal of H$_2$O$_2$ is conducted predominately via the OH·-generating Fenton reaction. Hydroxyl radical is considered to be the most notorious reactive oxygen species due to its high reactivity (about 10$^6$ times less stable compared to H$_2$O$_2$)$^{36}$. However, for the same reason, extracellularly-produced hydroxyl radical is incapable of reaching sensitive intracellular targets$^{44}$. Most hydroxyl radicals are buffered by extracellular proteins and some by Asc and membrane lipids. Oxidised lipids are reduced by Asc/tocopherol coupled antioxidant activity$^{37}$. Under such settings reactive oxygen species remain in the extracellular compartment
leaving cancer cells generally unharmed. It should be stressed out that presented mechanisms are not dependent on cancer cells and types, as documented by using two lines with different metastatic potentials and ontogenetically different non-cancer cells.

It has been shown recently that HIF-1α-signalling cascade is essential for cancer cells' resistance to ascorbate-provoked cytotoxicity. The level of inducible subunit of HIF-1 (HIF-1α) is regulated by hydroxylation, which is performed by HIF-1 prolyl hydroxylases (HPH), leading to HIF-1α degradation. HPH show a complex net of regulators that involves Asc, Fe²⁺, and O₂, which are required for HPH activity, as well as reducing agents (such as thiols that activate these enzymes), different glucose metabolites, and H₂O₂. Several studies have shown that the supplementation of Asc to cultured cancer cells provokes a drop in HIF-1α level. However, we found that Asc provoked an increase in HIF-1α level in LNCaP whereas it did not exert significant effects in PC-3 cells. A plausible explanation is in the opposing effects of Asc and H₂O₂ on the activity of HPH. Namely, in contrast to Asc, H₂O₂ is known to inhibit HPH and to provoke an increase in the HIF-1α level. It is tempting to speculate that H₂O₂-provoked inhibition of HPH prevailed over the activating effects of Asc in LNCaP cells, which showed low constitutive HIF-1α level and in relation to this a probable high HPH activity. Hydrogen peroxide-mediated inhibition of HPH could take place either via intracellular oxidation of Fe²⁺ to Fe³⁺ or via a decrease in the intracellular level of thiols and Asc. In PC-3 cells, constitutive HIF-1α level was high (as noted previously), so inhibitory effects of H₂O₂ on HPH could not be exerted/observed, and/or the opposing effects of Asc and H₂O₂ were balanced. Of note, high constitutive HIF-1α on HPH could not be exerted/observed, and/or the opposing effects of Asc and H₂O₂ were balanced. Of note, high constitutive HIF-1α on HPH could not be exerted/observed, and/or the opposing effects of Asc and H₂O₂ were balanced.

Figure 5 | The level of HIF-1α in cancer cells exposed to Asc (5 mM) and Fe (5 μM). (a) LNCaP. (b) PC-3. Micrographs showing HIF-1α immunofluorescence and HIF-1α merged with DAPI staining of nuclei, average per-pixel intensities of HIF-1α immunofluorescence (bars not sharing a common letter are significantly different and percentage distribution of cells according to average per-pixel intensities of fluorescence are presented (the number of cells (%)) was bracketed to 10 AU steps). Cells were stained 2 h after the treatment. Statistical analysis was performed using one-way ANOVA with post hoc Duncan test. All statistical differences were at the 0.001 level.

Although our experimental setup took into account the total concentration of iron in relevant biological milieu (plasma and interstitial fluid), redox activity of iron in cell culture media and in vivo is further complicated by a number of iron-binding molecules, such as transferrin and ferritin. The concentrations of transferrin and ferritin as well as their saturation with iron in FCS are not consistent, so their effects on redox activity of iron in cell culture media are hard to estimate. Pertinent to this, it was essential to examine the effects of Asc on cancer cells in biological fluid. The absence of anticancer effects in human plasma implies that our setup (medium supplemented with iron) properly reflects in vivo redox milieu. In addition, anticancer effects gradually decreased in cell culture medium with increasing volume percent concentration of FCS, i.e. with increasing resemblance of the applied medium with biological fluid. It can be further argued that the redox activity of the Asc/Fe system in interstitial fluid, which is the main site of Asc’s anticancer activity in vivo, could be different compared to plasma, since these differ in composition (for example in their proteome). However, it has been documented that interstitial fluid and plasma show similar capacities to prevent iron-dependent oxidation. It is noteworthy that the problem of unsuitability of common cell culture media for redox research, which clearly emerged in this case, have been outlined also by others and might affect the performance of a number of compounds other than Asc.

The Pauling/Cameron-Creagan/Moertel “conflict” about the applicability of oral Asc in cancer treatment that went on throughout the 1970’s and the 1980’s ended in disappointment. As a matter of fact, the concept of oral Asc application suffered from a major error at the very beginning. Namely, the bioavailability of ingested Asc is limited by the refractory response – a set of mechanisms that our
organism uses in order to maintain flexible redox poise and normal 
redox signalling. The findings of Levine and co-workers that even 
 mega doses of oral Asc cannot raise its level in the blood over modest 
200 μM pointed out the problem. This instigated renewed interest 
in the application of Asc in cancer treatment, but this time using 
intravenous administration in order to bypass limited intestinal 
asorption and to reach millimolar Asc concentrations in the blood 
and interstitial fluid. This was followed by somewhat spectacular 
results of a large number of in vitro studies which showed that cancer 
cells are efficiently killed by pharmacological Asc acting as a pro-
drug for H2O2 production. Unfortunately, it appears that research of 
the anticancer effects of Asc suffered from systematic errors once 
again and that we are on the verge of yet another disappointment. In 
addition to findings presented here, other evidence predict a grim 
future for Asc-based cancer therapy. For example, Asc shows rela-
tive limited efficiency in animal tumour models. Pertinent to this, 
we employed tumour spheroid model which shows complexity that 
is between in vivo tumours and monolayer cultures, and which is 
known to be more resistant to anticancer agents compared to the latter. 
Only weak tumour static effects were observed implying that 
Asc has already slipped from the group of potential anticancer drugs to a category of chemotherapy adjuvants.

Methods

Cells. Human androgen-dependent and androgen-independent prostate cancer cell lines, LNCaP and PC-3, were kindly gift from Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Cells lines were validated by short 
tandem repeat DNA fingerprinting, and matched known American Type Culture 
Collection (ATCC, Rockville, USA) fingerprints. Cells in logarithmic growth phase were cultured in RPMI-1640 medium supplemented with 10% (v/v) FCS (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 0.01% sodium pyruvate and 
antibiotics (culture medium) (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C in a 
humidified atmosphere with 5% CO2. After standard trypsinisation, cells were seeded 
in flat-bottomed 96-well plates (Sarstedt, Nümbrecht, Germany) (7×103 cells/well) for other measurements. Astrocytes were isolated from mixed 
glial cell cultures prepared from brains of newborn Dark Agouti rats as previously 
described. Astrocytes were grown in the above-mentioned culture medium 
supplemented with 4 mL glucose. They were purified by repetition of trypsinisation (0.25% trypsin and 0.02% EDTA, both from Sigma-Aldrich) and re-plating. The cells 
used in these experiments were obtained after the third passage. Astrocytes were 
seeded at 1.5×106 cells/mL/well in 24-well plates.

Treatment. Cells were incubated for 2 h in RPMI-1640 with 10% FCS or in undiluted human plasma (collected on the day of experiment), with or without 1-ascorbic acid (Sigma-Aldrich) at final concentrations of 5 or 10 mM. Two hour incubation period mimics clinical pharmacokinetics and has been applied previously in similar in vitro 
studies. Ascorbate stocks were buffered to pH 7.0 with NaOH and prepared 
immediately before use. Iron was added to cell culture medium at final concentrations 
of 0, 5, 10, 15, and 30 μM in the form of FAC (17% iron content, Serva, Heidelberg, Germany) or FeCl3 (Sigma-Aldrich). Treatment with H2O2 (200 μM; Carlo Erba Reagents, Milano, Italy) was performed in the absence or the presence of Asc (5 mM) and/or FAC (5 μM). Plasma samples were obtained from six healthy volunteers 
between the ages of 30 and 45 years (m/f = 3/3), using Vacutainer tubes containing 
Li-heparin as the anticoagulant per 4 mL of blood (BD, Franklin Lakes, NJ, USA) 
and centrifugation (2000 g/10 min/4 °C). A tourniquet was applied to the upper arm.

Figure 6 | Effects of pharmacological Asc on the size of tumour 
spheroids. (a) Images of LNCaP spheroids obtained at 10X and 2X 
magnification. (b) The effects of Asc (5 mM) and Fe (5 μM) on spheroid 
growth. (c) The effects of Asc (10 mM) and Fe (5 μM) on spheroid growth. 
Area of each sphere was normalized to its area at 0 h (at the start of the 
treatment). a - significant compared to initial area (0 h); b - significant 
compared to area at 4 h. Statistical analysis was performed using one-way 
ANOVA with post hoc Duncan test.

Figure 7 | Redox activity of Asc/Fe system. (a) In cell culture media. 
(b) Under in vivo settings with physiological iron concentration. Asc reduces Fe3+ to Fe2+, which react with O2 in order to produce superoxide. 
The latter is dismutated to H2O2, which can affect cancer cells or be decomposed, depending on iron concentration. The decomposition is 
mediated via Fenton reaction and the oxidation of extracellular proteins 
and Asc. It is important to note that in vivo iron is not free but bound to 
hydroxyl, phosphate ions, small ligands or proteins (not presented). Asc - 
ascorbic radical; CG - carbonyl groups; O2 - superoxide radical anion; 
OH - hydroxyl radical.

Nevertheless, a number of clinical trials are underway and our find-
ings could be used in order to adjust current therapeutic approaches. 
Co-application of iron chelators in order to reduce redox active iron 
to a level that promotes the anticancer activity of Asc appears to be 
realistic. Iron chelation has shown significant in vitro and in vivo 
anticancer effects irrespective of Asc (most likely by causing iron 
deficiency). There are still many problems with iron chelation ther-
rapy, such as limited bioavailability, misdosing and toxicity, but in 
order to promote Asc’s anticancer effects, less aggressive agents, such 
as disulfiram, might be sufficient.
Viability assays. Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-
2Htetrazolium bromide (MTT; Sigma-Aldrich) to formazan reflects mitochondrial
activity and viability of cultured cells, while the number of adherent, viable cells
 correlates with crystal violet (CV) (Mol, Belgrade, Serbia) staining. Following 2 h
incubation period, supernatants were collected for EPR measurements, while cells
were washed with PBS and resuspended in cell culture medium for additional 24 h.
To evaluate mitochondrial respiration, MTT solution was added to cell cultures at the
final concentration 1 mg/mL, incubated for 4 h after which produced formazan
was dissolved in DMSO. In CV assay, adherent cells were first fixed with 4% PFA for
10 min and subsequently stained with 1% CV for 15 min at room temperature. The
cells were then washed, dried, and dye was dissolved in 33% acetic acid.
Mitochondria-dependent production of formazan and the intensity of absorbed CV
by adherent cells were assessed with an automated microplate reader at 570/640 nm.
Cell viability was expressed relative to control (untreated cells; same experimental
day).
Biochemical assays. Following 2 h incubation, with or without iron (5 μM) and/or
Asc (5 mM), supernatants were collected, while cells were washed 3 times with PBS,
snap frozen in liquid N2, and stored at −80°C. Cells were detached with a Teflon
cell scraper, dissolved in extraction buffer and centrifuged at 30000 g for
90 min. Intracellular content of thiol groups was determined according to Ellman.70
Total concentration of intracellular iron was determined according to the method of
Bad et al.55. Protein concentration was determined by the method of Bradford56, 24-
Dinitrophenylhydrazine was used for determining carbonyl content in supernatants
according to Levine et al.72. The carbonyl content was calculated from the absorbance
measurement at 380 nm and an absorption coefficient of 22000 M−1 cm−1. The level
of malondialdehyde (MDA) was measured according to procedure of Ohkawa et al.57
The concentration of iron in the plasma and medium was determined according to the
method of Bouda.110 CAT activity in plasma was determined using two previously
described methods57,71.
EPR measurements. Samples were placed in Teflon tubes with a wall thickness of
0.025 mm and an internal diameter of 0.6 mm (Zeus Industries, Raritan, NJ, USA)
and insulating inserts. EPR spectra were recorded on a Bruker ESR spectrometer operating at
X-band (9.51 GHz), and EW software (Scientific Software Inc., Bloomingtom, IL, USA).
EPR signal of ascorbyl radical in supernatants was recorded using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 20 mW. Simulation and double integration of EPR spectra of ascorbyl radical were performed in order to determine intensities of
EPR signal of ascorbyl radical, using WINEPR SimFonia Computer Program (Bruker
Analytische Messtechnik GmbH, Karlsruhe, Germany) and simulation parameter:
aH = 2.1. The concentration of ascorbyl radical was determined using calibration curve
that was prepared with 3-carboxypropyl (Sigma-Aldrich) as a standard.77 For the EPR
assays in cell cultures cells were trypsinized, washed twice in PBS, adherent
PBAs at a density of 1.5 × 105 cells/100 μL and placed on ice. Thiol-sensitive biradical
spin probe RSSR (Enzo Life Sciences International, Plymouth Meeting, PA, USA) was
dissolved in DMSO and added to a final concentration of 100 μM. EPR spectra were recorded after
4 min incubation, using the following settings: modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 10 mW. The concentration
of SH groups was determined using calibration curve that was prepared with
glutathione as a standard for intracellular thiols.
Oximetry. The O2 concentration and the rate of oxygen consumption/production
(d[O2]/dt) was determined using a Clark electrode – OXILP Dissolved Oxygen
Package (Qubit Systems Inc, Kingston, ON, Canada) operating with Logger Pro 1
software (Vernier, Beaverton, OR, USA). Experiments were performed in RPMI-1640
+ 10% (v/v) FCS with or without the addition of Asc (5 mM) and/or FAC (5 μM). All
systems were recorded for 5 min before the addition of Asc and/or Fe, in order to
establish the stability of baseline and zero rate of O2 change (not shown).
Accumulation of H2O2 was determined by measuring O2 production induced by the
addition of CAT to the system 58. Although various other assays and probes for H2O2
were evaluated, the assay described here was selected because it is well
defined unlike the cross-section area of the whole cell.
Tumour spheroids. Spheroids were formed using a modified method of Ivascu and
Kubbe66. In brief, 1 × 105 LNCaP cells were suspended in 100 μL of ice-cold
medium and placed in standard polystyrene 96-well plate with a round bottom
(Sarstedt, Nürnberg, Germany; Prod. No. 82.1582.001). Matrigel® (BD Labware,
Bedford, MA, USA) was applied at 2.5% (v/v) using ice-cold pipette tip. Spheroid
formation was initiated by centrifugation of the plates at 1000 g for 10 min. The plates
were incubated under standard cell culture conditions for 2 days, after which
compact spheroid morphology was evident. The number of spheroids was 10–20 per
well. It is noteworthy that previously described procedure for formation of a single
spheroid used poly-HEMA–coated plates and a lower number of LNCaP cells per mL
(1 × 104 cells in 200 μL).66 Spheroids were further untreated or treated with Asc
(5 mM or 10 mM), Fe (5 μM), or with both Asc and Fe. Final volume per well was
200 μL. Images of spheroids were acquired immediately after, and 4 h and 24 h after
the beginning of the treatment, using Zeiss Axiosvert microscope and magnifica Leica
MZ16 (Leica Microsystems, Heerbrug, Switzerland). Cells were treated for 24 h. The
treatment could not be stopped, i.e. medium change was not possible, because it
would result in the movement and/or decomposition of spheroids. For each
treatment, cross-section areas of 20 spheroids with distinct round morphology
(randomly selected in three wells) were followed up and measured at three time points
using Image J. The area of each spheroid at 4 h and 24 h was normalized to the area
at the beginning of the treatment.
Statistics. All experiments were performed at least three times on separate
experimental days. The data are presented as mean ± standard deviation (S.D.).
Statistically significant differences between the means obtained in different experimental
settings were evaluated by the means of ANOVA (two-way or one-way as appropriate) with
post hoc Duncan’s range test (p < 0.05) using STATISTICA 8.0 (StatSoft Inc, Tulsa,
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Author contributions
I.S. and D.M.I. designed the research; M.M., J.B.P., M.S., D.M.I., S.M., and I.S. performed the experiments; I.S., J.B.P., M.M., and M.S. analysed the data. I.S., D.J., M.M., J.B.P., and D.M.I. wrote the paper.

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