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

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In vitro studies have shown that hydrogen peroxide (H_2O_2) 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 H_2O_2. The latter is mediated by Fenton reaction and prevents H_2O_2 accumulation. The hydroxyl radical, which is produced in the Fenton reaction, is buffered by extracellular proteins, and could not affect intracellular targets like H_2O_2. 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 tolerated[1–3]. 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 unaffected[4,5]. Furthermore, the mechanisms of Asc's anticancer activity were described by Chen and co-workers[6], and later confirmed by others[7,8], showing that Asc is a pro-drug for extracellular generation of hydrogen peroxide (H_2O_2) and that iron is essential for H_2O_2 production. In brief, Asc reduces iron (Fe^{3+} to Fe^{2+}), which further reacts with molecular oxygen forming the superoxide radical anion. Superoxide undergoes (non)enzymatic dismutation to produce H_2O_2. Supra-physiological concentrations of H_2O_2 enter the cell and exert cytotoxic effects. Both apoptotic and necrotic pathways of cell death were implicated, with necrosis more prevalent at increasing Asc doses[4].

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[9], while another group found ~3 μM Fe in RPMI-1640 with 10% (v/v) FCS[10]. 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 cytotoxicity[5]. 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 μM[11], and even higher in cancer patients[12,13], as well as in patients receiving
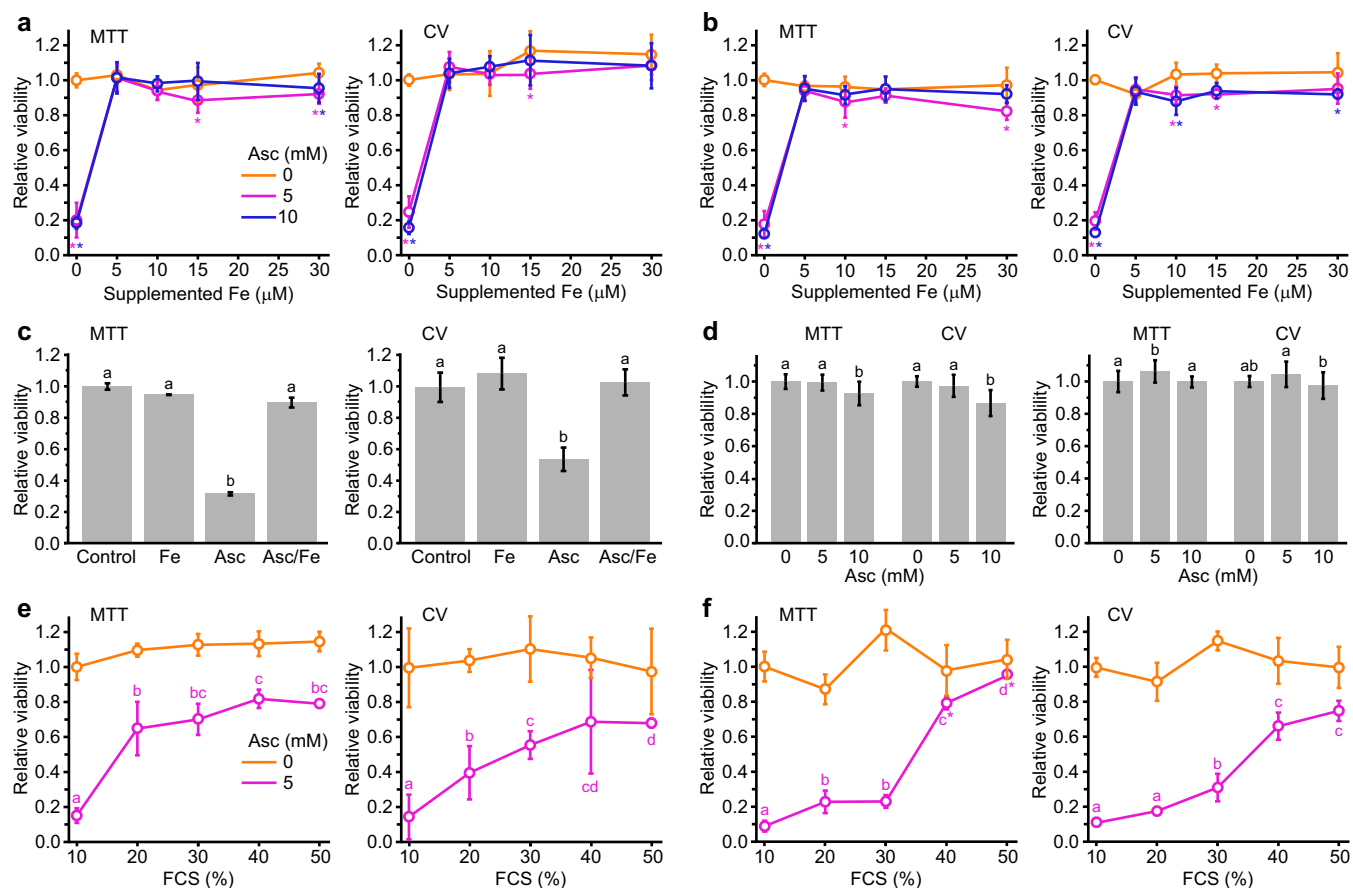
Figure 1 | Effects of pharmacological ascorbate and physiological iron on the viability of cancer and primary cells according to MTT and CV assays.
(a) LNCaP and (b) PC-3 cells treated in cell culture medium (RPMI-1640 + 10% FCS) with different concentrations of supplemented iron; * - statistically significant (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.

chemotherapy[14]. The concentration of iron in interstitial fluid (outside the CNS) closely mirrors that found in the plasma[11,15], whereas iron appears to accumulate in tumour interstitial fluid[16]. In addition, Asc may provoke the release of iron from extracellular stores, such as ferritin which could be actively produced and released by cancer cells[7]. It is important to point out that although plasma and interstitial fluid share very similar redox properties[17], the critical *in vivo* accumulation of H_2O_2 could take place only in interstitial fluid, as catalase (CAT)-rich erythrocytes buffer H_2O_2 in the blood. The lack of studies that address the effects of iron at (patho)physiological concentrations is rather perplexing, particularly if we take into account previous findings showing that iron can protect cancer cells from H_2O_2[18].

Herein we study the anticancer/cytotoxic activity of Asc under conditions that reflect *in vivo* settings: (i) in cell culture medium with (supplemented) iron at concentrations that are comparable to those in plasma and interstitial fluid; (ii) in human plasma; and (iii) using tumour spheroid model that mimics biological properties of micro-metastases. We use two human prostate cancer cell lines – LNCaP (low metastatic potential, androgen-dependent) and PC-3 (high metastatic potential, androgen-independent), as well as primary astrocytes. Astrocytes were selected for comparison with cancer cells because they are: (i) ontogenetically different (ectodermal origin) compared to prostate cells (endodermal origin): (ii) acquired from a different species; and (iii) susceptible to Asc cytotoxicity[19]. Biochemical assays, oximetry, electron paramagnetic resonance (EPR),

spectroscopy, and immunofluorescence are employed in order to elucidate the interactions between Asc and iron.

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 $FeCl_3$ 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
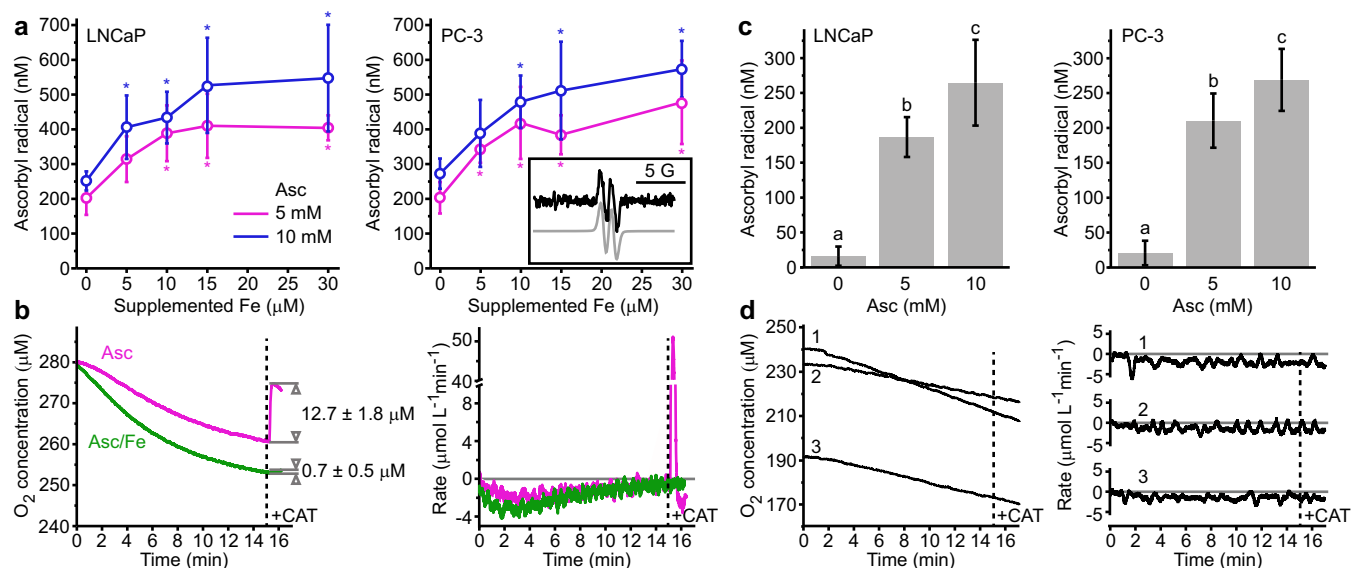
Figure 2 | The effects of ascorbate and iron on hydrogen peroxide production in cell culture medium and human plasma. (a) Concentration of ascorbyl radical (marker of H_2O_2 production) in cell culture medium samples that were collected after incubation of LNCaP or PC-3 cells for the purposes of viability 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 supplemeted). (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_2O_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_2O_2 production in this system[22]. The level of H_2O_2 was estimated by the addition of CAT which resulted in a rapid and transient O_2 production from H_2O_2 breakdown (Fig. 2b). The amount of O_2 produced and the rate of O_2 production were much smaller/lower 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_2O_2 molecules for each O_2 produced ($2H_2O_2 \rightarrow 2H_2O + O_2$), the steady-state concentration of H_2O_2 in the presence of cell culture medium iron is estimated to be around 25 μM. Physiological iron reduced the level of H_2O_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_2O_2. Ascorbyl radical is produced in the plasma supplemented with Asc (Fig. 2c), implying that Asc is redox active and that H_2O_2 is produced in this system. Ascorbyl radical concentrations found in our experiments correspond to levels in the plasma of cancer patients receiving Asc intravenously[23]. Polarographic measurements on plasma resemble data obtained on cell culture medium with physiological concentration of Fe. Only traces of H_2O_2 could be detected in plasma supplemented with Asc (Fig. 2d). Altogether these results show that in the presence of pharmacological Asc, physiological Fe promotes H_2O_2 decomposition, thus not allowing H_2O_2 accumulation. In the presence of cell culture medium concentration of iron, H_2O_2 accumulates because its degradation is very slow or completely absent.

Fig. 3a 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_2O_2-provoked oxidation[24]. This is in line with previous findings[8,25]. However, in the presence of iron at physiological concentration, the Asc-mediated decrease in the level -SH groups was abolished, *i.e.* iron prevented intracellular oxidation. Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + ^•OH$) appears as a plausible mechanism of removal of extracellular H_2O_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
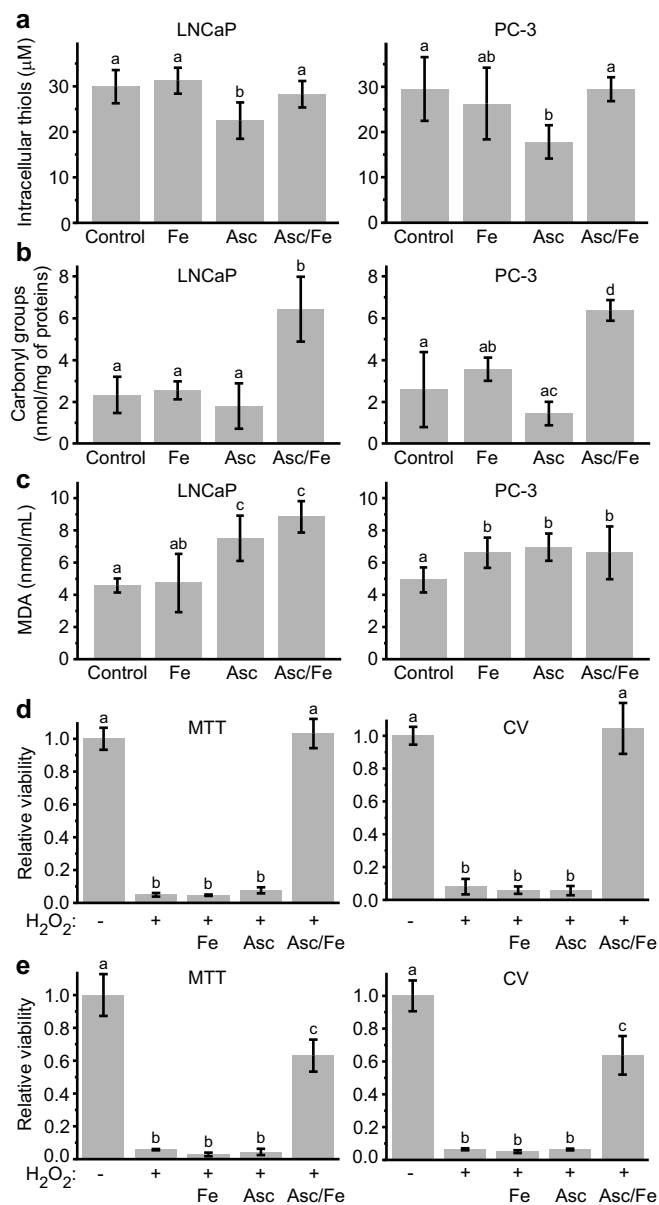
Figure 4 | Intracellular iron in LNCaP and PC-3 cells. Cells were exposed to Asc (5 mM) and/or Fe (5 or 30 μM). The results are normalized to 1×10^6 cells/mL. Statistical analysis was performed using one-way ANOVA with *post hoc* Duncan test. There was no significant difference ($p < 0.05$) in concentration between any of the treatments.

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_2O_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_2O_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 florescence 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_2O_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_2O_2 compensates for H_2O_2 generation and prevents its accumulation (Fig. 7b). The removal of H_2O_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 10^9 times less stabile compared to H_2O_2)[36]. However, for the same reason, extracellularly-produced hydroxyl radical is incapable of reaching sensitive intracellular targets[18]. Most hydroxyl radicals are buffered by extracellular proteins and some by Asc and membrane lipids. Oxidised lipids are reduced by Asc/tocopherol coupled antioxidative activity[37]. Under such settings reactive oxygen species remain in the extracellular compartment

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Figure 3 | The effects of ascorbate and iron on redox settings in cancer cell culture. (a) Intracellular thiols in LNCaP and PC-3 cells. The results are normalized to 1×10^6 cells/mL. (b) Extracellular carbonyl groups (marker for oxidized proteins) and (c) MDA (marker for oxidized lipids) in supernatants that were collected following incubation of LNCaP and PC-3 cells. (d) Viability of LNCaP and (e) PC-3 cells exposed to H_2O_2 (200 μM) in the absence or the presence of Asc and/or Fe. Concentrations of supplemented Asc and Fe were 5 mM and 5 μM, respectively. Bars not sharing a common letter are significantly different ($p < 0.05$). Statistical analysis was performed using one-way ANOVA with *post hoc* Duncan test.

level of lipid peroxidation was not affected by iron supplementation (Fig. 3c). All these experiments have been also performed using 30 μM supplemented iron with very similar results. Altogether this implies that iron at physiological concentration and pharmacological Asc propel the production of •OH, most of which are removed by protein oxidation. It is important to point out that we have applied EPR spin-trapping spectroscopy in order to directly detect the production of •OH. However, in line with our previous experience, spin-trapping in Asc-rich systems is unfeasible because Asc reduces spin-adducts. Finally we tested the ability of pharmacological Asc and physiological Fe to protect cells from exogenous H_2O_2 (Fig. 3d, e). It can be observed that Asc in the presence of cell culture medium
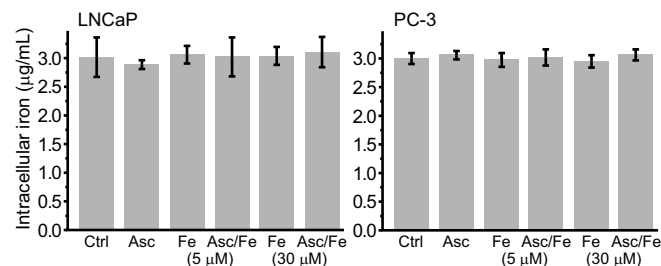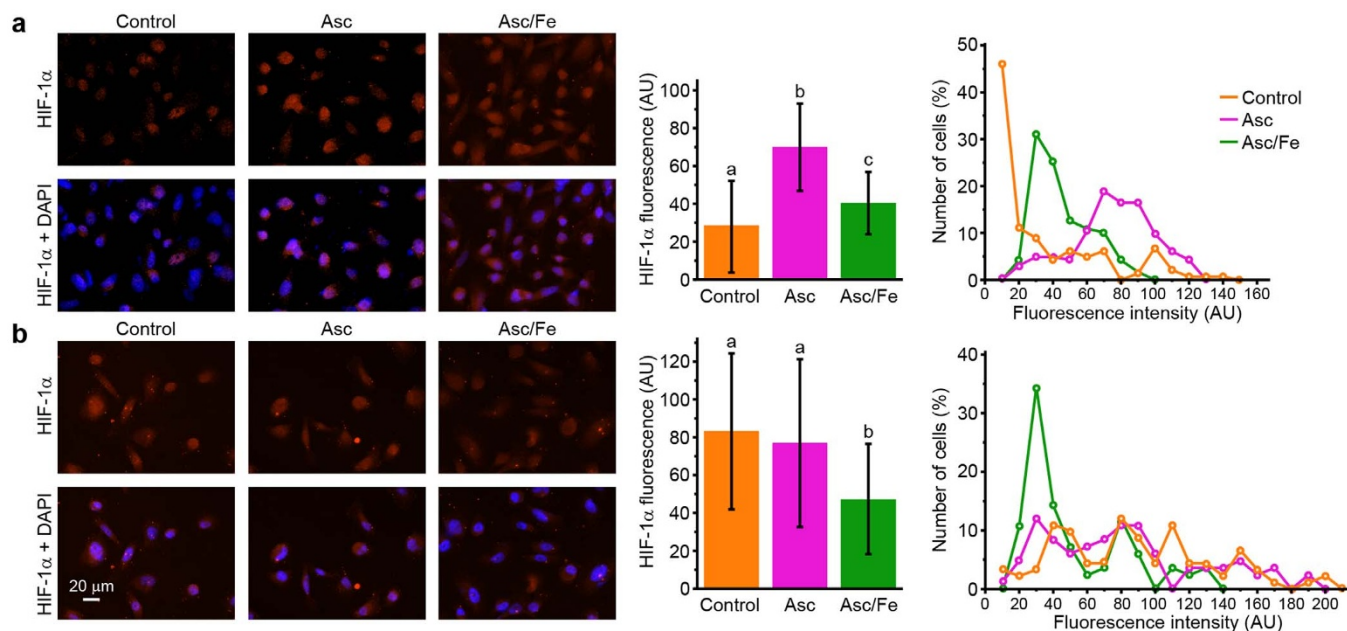
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Figure 5 | The level of HIF-1α in cancer cells exposed to Asc (5 mM) and Fe (5 μM). (a) LNCaP. (b) PC-3. Micrographs showing HIF-1α immunofluorescence and HIF-1α merged with DAPI staining of nuclei, average per-pixel intensities of HIF-1α immunofluorescence (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.

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[31]. 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, Fe^{2+}, and O_2, which are required for HPH activity, as well as reducing agents (such as thiols that activate these enzymes), different glucose metabolites, and H_2O_2[34]. Several studies have shown that the supplementation of Asc to cultured cancer cells provokes a drop in HIF-1α level[32–34]. 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 H_2O_2 on the activity of HPH. Namely, in contrast to Asc, H_2O_2 is known to inhibit HPH and to provoke an increase in the HIF-1α level[35]. It is tempting to speculate that H_2O_2-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 Fe^{2+} to Fe^{3+} 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[33]), so inhibitory effects of H_2O_2 on HPH could not be exerted/observed, and/or the opposing effects of Asc and H_2O_2 were balanced. Of note, high constitutive HIF-1α levels have been observed in many highly glycolytic cancer cells, and it appears to be related to pyruvate- and oxaloacetate-mediated inhibition of HPH[34]. The setup changed in the presence of physiological iron. In LNCaP, Asc-provoked increase was significantly reduced, whereas in PC-3 the level of HIF-1α felt below baseline. These effects are most likely related the ability of physiological iron to prevented Asc-provoked H_2O_2 accumulation. Under such settings, HIF-1α lowering effects of supplemented Asc most likely emerged. It is worth mentioning that the supplementation of iron did not

increase the intracellular iron concentration, however Asc might maintain iron in Fe^{2+} in the intracellular compartment thus increasing HPH activity. Altogether these phenomena are interesting, but appear only to reflect redox settings in our system. In other words, a decrease in the level of pro-survival factor in cells that were protected from the cytotoxic effects of Asc appears not to be directly involved in the mechanisms of protection.

Although our experimental setup took into account the total concentration of iron in relevant biological milieus (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 feritin[38]. The concentrations of transferrin and ferritin as well as their saturation with iron in FCS are not consistent[9], so their effects on redox activity of iron in cell culture medium 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[39]). However, it has been documented that interstitial fluid and plasma show similar capacities to prevent iron-dependent oxidation[17]. It is noteworthy that the problems 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[40].

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[41]. 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
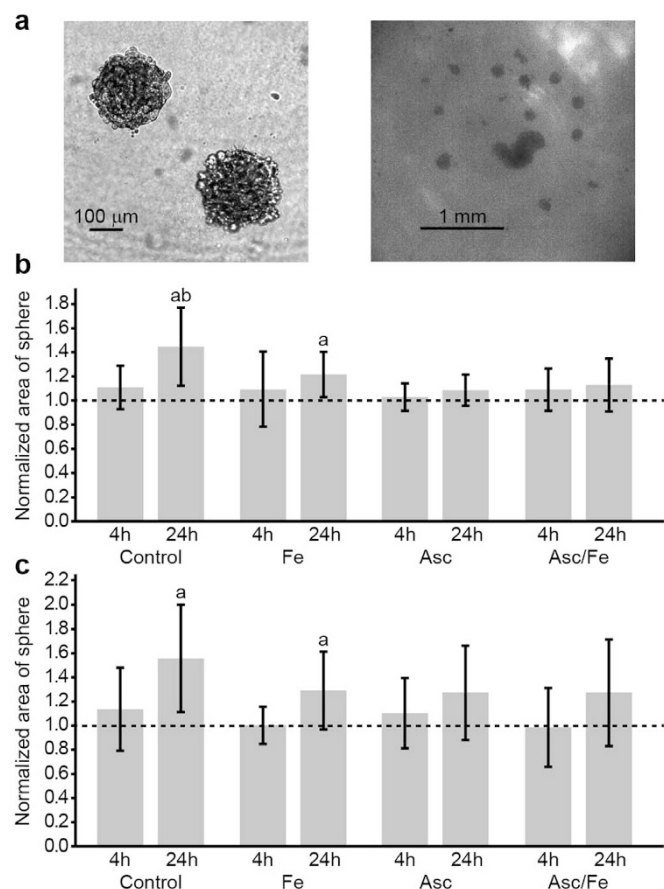
Figure 6 | Effects of pharmacological Asc on the size of tumour spheroids. (a) Images of LNCaP spheroids obtained at 10× and 2× magnification. (b) The effects of Asc (5 mM) and Fe (5 μM) on spheroid growth. (c) The effects of Asc (10 mM) and Fe (5 μM) on spheroid growth. Area of each sphere was normalized 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.

organism uses in order to maintain flexible redox poise and normal redox signalling[36]. 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[42]. This instigated renewed interest in the application of Asc in cancer treatment, but this time using intravenous administration in order to bypass limited intestinal absorption and to reach millimolar Asc concentrations in the blood and interstitial fluid[4,5]. 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 H_2O_2 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 relatively limited efficiency in animal tumour models[43]. 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[44]. Only weak tumour static effects were observed implying that Asc-based therapy might suffer from more than one shortcoming. In addition, completed phase I clinical trials showed no objective anticancer response to treatment with pharmacological Asc[1–3], and it appears that Asc has already slipped from the group of potential anticancer drugs to a category of chemotherapy adjuvants[45,46].
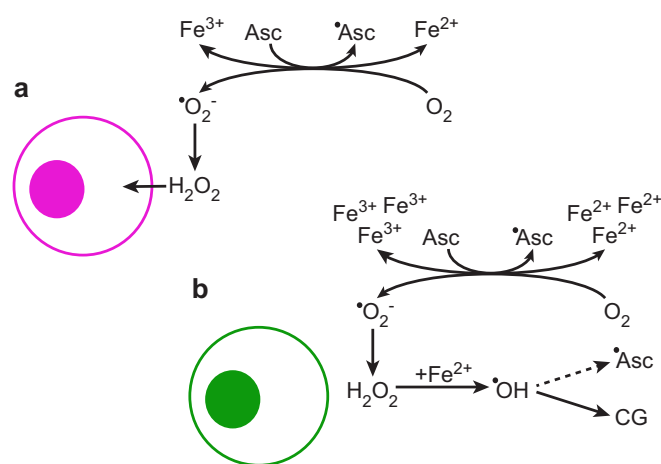

Figure 7 | Redox activity of Asc/Fe system. (a) In cell culture media. (b) Under *in vivo* settings with physiological iron concentration. Asc reduces Fe^{3+} to Fe^{2+}, which react with O_2 in order to produce superoxide. The latter is dismutated to H_2O_2, which can affect cancer cells or be decomposed, 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). $^{\bullet}$Asc - ascorbyl radical; CG - carbonyl groups; $^{\bullet}O_2^-$ - superoxide radical anion; $^{\bullet}OH$ - hydroxyl radical.

Nevertheless, a number of clinical trials are underway and our findings 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)[47]. There are still many problems with iron chelation therapy, such as limited bioavailability, misdosing and toxicity[38], but in order to promote Asc's anticancer effects, less aggressive agents, such as disulfiram, might be sufficient[48].

Methods

Cells. Human androgen-dependent and androgen-independent prostate cancer cell lines, LNCaP and PC-3, were a kind gift from Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Cell 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% CO_2. After standard trypsinisation, cells were seeded in flat-bottomed 96-well plates (Sarstedt, Nümbrecht, Germany) (7×10^3 cells/well for LNCaP and 5×10^3 cells/well for PC-3) for viability determination, or in 6-well plates (3×10^5/well) for other measurements. Astrocytes were isolated from mixed glial cell cultures prepared from brains of newborn Dark Agouti rats as previously described[49]. Astrocytes were grown in the above-mentioned culture medium supplemented with 4 g/L 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 1.5×10^5 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 L-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 $FeCl_3$ (Sigma-Aldrich). Treatment with H_2O_2 (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

during sampling from antecubital vein and sampling was completed within 90 s following the tourniquet application. A special care was made in order to prevent haemolysis or the collection of erythrocytes or buffy coat. All procedures related to the use of human blood in this study are in conformity with the recommendation provided in The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The protocols were approved by the local Ethics Committee. Viability experiments were performed in triplicate for each plasma sample. Iron was not supplemented to plasma samples.

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 of 0.5 mg/mL, incubated for 1 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 N_2 and stored at −80°C. Frozen 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[50]. Total concentration of intracellular iron was determined according to the method of Rad et al.[51]. Protein concentration was determined by the method of Bradford[52]. 2,4-Dinitrophenylhydrazine was used for determining carbonyl content in supernatants according to Levine et al.[53]. 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.[54]. The concentration of iron in the plasma and medium was determined according to the method of Bouda[55]. CAT activity in plasma was determined using two previously described methods[56,57].

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 inserted into quartz capillaries. EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz), and EW software (Scientific Software Inc., Bloomington, 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: a^H = 2.1. The concentration of ascorbyl radical was determined using calibration curve that was prepared with 3-carboxyproxyl (Sigma-Aldrich) as a standard[37]. For the EPR assay for -SH groups[36], cells were trypsinized, washed twice in PBS, resuspended in PBS at a density of 1.5×10^5 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 O_2 concentration and the rate of oxygen consumption/production (d[O_2]/dt) was determined using a Clark electrode – OX1LP 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 O_2 change (not shown). Accumulation of H_2O_2 was determined by measuring O_2 production induced by the addition of CAT to the system[58]. Although various other assays and probes for H_2O_2 quantification are available, most of them are likely to be interfered by millimolar concentrations of Asc[59]. CAT (600 U; Sigma-Aldrich C1345) was added at the point when systems approached a zero rate of O_2 consumption. Polarographic measurements were also performed on plasma samples of three volunteers. CAT was supplemented 15 min after the application of Asc (5 mM).

HIF-1α immunofluorescence and quantification. Cells were seeded in glass bottom chambers (Lab-Tek® II Chamber Slide™, Thermo Fisher Scientific, Waltham, MA, USA) at 3 × 10⁴ cells/chamber overnight. Cells were treated with Asc (5 mM) or Asc + Fe (5 μM) for 2 h, washed and resuspended in cell culture medium for additional 2 h at 37°C. Cells were fixed with 4% PFA at 4°C for 15 min and permeabilized in Triton X-100 (0.25%) for 10 min, which was followed by the blocking of non-specific

staining with FCS (10%) for 30 min. Then, cells were stained with mouse anti-HIF-1-alpha [H1alpha67] (Abcam, Cambridge, UK) (1/200) antibody overnight at 4°C, and visualized with fluorophore-labelled secondary antibody Alexa Fluor 555 donkey anti-mouse (Life Technologies - Invitrogen, Carlsbad, CA, USA) (1/500). Cell nuclei were counterstained with DAPI. Images were acquired using Zeiss Axiovert microscope (Carl Zeiss, Jena, Germany). Fluorescence measurements were performed using Image J (NIH, USA), as described previously[60]. Mean per-pixel fluorescence intensity of HIF-1α in the cross-section area of nucleus of each cell was evaluated and presented as arbitrary units (AU). In addition, the percentage distribution of cells showing fluorescence intensities within specific range (10 AU steps were used, e.g. 0–10 AU, 10–20 AU, etc) was established. The whole range of fluorescence intensities (0–255 AU) was covered. It is important to note that HIF-1α was not specifically located in nuclei, but this area 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 Kubbies[61]. In brief, 1 × 10⁴ LNCaP cells were suspended in 100 μL of ice-cold medium and placed in standard polystyrene 96-well plate with a round bottom (Sarstedt, Nümbrecht, 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)[61]. 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 Axiovert microscope and magnifier Leica MZ16 (Leica Microsystems, Heerbrugg, 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.). Statistical differences between the values 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, OK, USA).

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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.

Additional information

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