The ROS-induced cytotoxicity of ascorbate is attenuated by hypoxia and HIF-1alpha in the NCI60 cancer cell lines

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Abstract

Intravenous application of high-dose ascorbate is used in complementary palliative medicine to treat cancer patients. Pharmacological doses of ascorbate in the mM range induce cytotoxicity in cancer cells mediated by reactive oxygen species (ROS), namely hydrogen peroxide and ascorbyl radicals. However, little is known about intrinsic or extrinsic factors modulating this ascorbate-mediated cytotoxicity. Under normoxia and hypoxia, ascorbate IC50 values were determined on the NCI60 cancer cells. The cell cycle, the influence of cobalt chloride-induced hypoxia-inducible factor-1alpha (HIF-1alpha) and the glucose transporter 1 (GLUT-1) expression (a pro-survival HIF-1alpha-downstream-target) were analysed after ascorbate exposure under normoxic and hypoxic conditions. The amount of ascorbyl radicals increased with rising serum concentrations. Hypoxia (0.1% O2) globally increased the IC50 of ascorbate in the 60 cancer cell lines from 4.5 ± 3.6 mM to 10.1 ± 5.9 mM (2.2-fold increase, P < 0.001, Mann–Whitney t-test), thus inducing cellular resistance towards ascorbate. This ascorbate resistance depended on HIF-1alpha-signalling, but did not correlate with cell line-specific expression of the ascorbate transporter GLUT-1. However, under normoxic and hypoxic conditions, ascorbate treatment at the individual IC50 reduced the expression of GLUT-1 in the cancer cells. Our data show a ROS-induced, HIF-1alpha- and O2-dependent cytotoxicity of ascorbate on 60 different cancer cells. This suggests that for clinical application, cancer patients should additionally be oxygenized to increase the cytotoxic efficacy of ascorbate.

Keywords: ascorbate • cancer • GLUT-1 • HIF-1alpha • hypoxia • ROS • therapy

Introduction

Despite its controversial history in cancer therapy in the 1970s and 1980s [1, 2], a large body of evidence has emerged in recent years supporting the hypothesis that high-dose ascorbic acid (AA, ascorbate, vitamin C) kills cancer cells in vitro and in vivo [3–6].

This cytotoxic effect is driven by the extra-cellular production of reactive oxygen species (ROS), e.g. hydrogen peroxide [7, 8], making high-dose ascorbate a pro-oxidative anticancer pro-drug. Present limitations to transfer this therapeutic concept to the clinics effectively are the high tissue concentrations needed to elicit a ROS-concentration above a toxic threshold. In this context, we recently demonstrated that patients afflicted with metastatic melanoma (clinical stage IV) have significantly lower plasma ascorbate levels compared with healthy controls and that polychemotherapy or immunotherapy further decreases plasma ascorbate levels in stage IV melanoma patients, and concluded that ascorbate substitution in physiological doses could be considered for late-stage melanoma patients [9]. In humans, the ascorbate concentration required to induce cytotoxicity in cancer cells can only be achieved via intravenous administration [9]. Ten- to 30 mM ascorbate serum peak concentrations are achievable through the i.v. administration of 25–100 g ascorbate [4]. In experimental tumour xenografts in mice, the intra peritoneal administration of 4 g ascorbate per kilogram of bw results in the accumulation of 20–40 mM ascorbate and 500 mM
ascorbate radical in the tumours [4]; however, it remains to be determined to which molarity ascorbate can be accumulated in malignant tumours and their metastases in human cancer patients. A large number of studies have observed an increased generation of ROS and the alteration of the redox status in cancer cells, which are more vulnerable to the increased oxidative stress induced by exogenous ROS-generating compounds that inhibit the endogenous antioxidant system [10]. In line, it was speculated that an exogenous increase in ROS stress in cancer cells might cause an elevation of ROS above a toxic threshold (thus overwhelming the antioxidant capacity of the cell), which possibly provides a biochemical basis to apply therapeutic strategies to selectively kill cancer cells by using ROS-mediated mechanisms [11].

In addition to having an altered ROS status, cancer cells and the tumour microenvironment are frequently hypoxic [12]. Hypoxia is a critical hallmark of solid tumours and involves enhanced cell survival, angiogenesis, glycolytic metabolism and metastasis [13–15]. Interestingly, a recent review on hyperbaric oxygen (HBO) treatment concluded that HBO can be inhibitory and reduce cancer growth in some cancer types [16].

In endometrial cancer, low ascorbate levels are associated with high hypoxia-inducible factor-1α (HIF-1α) activation, HIF-1α-mediated up-regulation of glucose transporter 1 (GLUT-1) and an aggressive tumour phenotype [17]. Most cells maintain ascorbate concentrations at low millimolar levels either by active transport via sodium-dependent vitamin C transporter (SVCT) that transport the reduced form of ascorbic acid or via the hexose transporter, GLUT-1, which transports dehydroascorbic acid (the oxidized form of ascorbic acid) competitively with glucose [18]. Thus, the level of GLUT-1 expression might be an indicator for the susceptibility of cancer cells towards ascorbate-induced cell death.

Regarding the currently observed limited efficacy of high-dose ascorbate on the killing of tumour cells in cancer patients in clinical phase I trials [10, 19], in the present study, we analysed whether hypoxic conditions might, in part, be responsible for cancer cell resistance towards ascorbate-induced cell death. In detail, we investigated whether (i) the ascorbate-induced generation of ROS was serum-dependent, (ii) the susceptibility to ascorbate-induced cytotoxicity in the 60 cancer cell lines of the NCI60 panel was merely influenced by exogenous O₂ availability and/or by HIF1α and (iii) correlated with the endogenous GLUT-1 expression. The cell lines of the NCI60 panel were chosen for this study as (i) they are well-defined and well-characterized (mutation status, RNA expression, etc.), (ii) they are the standard tool used for cytotoxicity testing in the screening of novel drugs by the NCI and (iii) as they consist of different tumour entities, thus enabling a detailed comparison.

Our results indicate that the generation of ROS from ascorbate is catalysed by serum and that severe hypoxia (as present in cancer metastases) mediates a reduced susceptibility (or resistance) of all 60 cancer cell lines towards ascorbate-induced inhibition of cell proliferation (driven by induction of apoptosis). This ascorbate resistance was enhanced by cobalt chloride (CoCl₂)-induced HIF1α [20]. However, the ascorbate-induced cytotoxicity did not correlate with the endogenous GLUT-1 expression. These data suggest that a clinical application of high-dose ascorbate on cancer patients should be complemented by oxygenation (e.g. via HBO treatment) of the patient to assure for adequate tissue oxygen levels necessary for ascorbate-driven ROS generation. This might improve the therapeutic anti-cancer properties of high-dose ascorbate in the cancer patient.

Materials and methods

Electron spin resonance spectroscopy (ESR)

To detect the induction of ascorbyl radicals by ascorbate, ESR was applied. Ascorbyl radical induction was measured in medium with 8 mM ascorbate. Electron spin resonance spectroscopy ascorbyl spectra were recorded as described previously [21]. Briefly, the samples were measured in a quartz flat cell (60 × 17 × 0.7 mm²) on a Bruker ESP300E x-band spectrometer (Rheinstetten, Germany) equipped with a TM 4103 resonator operating at 9.8 GHz. Instrument settings were as follows: modulation frequency 100 kHz, microwave power 5 mW, modulation amplitude, gain and time constant varied within the range of ascorbyl radicals. Electron spin resonance spectroscopy measurements were performed in triplicates.

Measurement of intracellular peroxide radicals (H₂O₂)

For the generation of peroxide radicals (H₂O₂), nine different cell lines were exposed to 8 or 16 mM ascorbate or to 0.5 mM H₂O₂, with or without the addition of 100 µg/ml catalase in RPMI full medium (refer to Cells and cell culture for details) for 1 hr. The medium was discarded, cells were washed once with PBS and H₂O₂ was measured in PBS by using dichlorofluorescein substrate (Invitrogen, Darmstadt, Germany) according to the procedure described by the supplier. Experiments were performed in quadruplicates.

Cells and cell culture

All 60 cell lines (NCI60 cells, Table 1, purchased from the National Cancer Institute, Bethesda, MD, USA), were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 1% penicillin and streptomycin and 1% L-glutamine. The medium was changed at 48 hrs intervals, and the cells were passaged upon 90% confluence. All cell cultures were maintained at 37°C in a 95% air/5% CO₂ atmosphere at 100% humidity. For hypoxia experiments, the normoxic O₂ level (21%) was reduced to 0.1% O₂ (hypoxia-incubator used: Galaxy 48R, New Brunswick [Eppendorf], Hamburg, Germany).

Ascorbate and CoCl₂ treatment

The following chemicals were used: Injectable vitamin C solution (Pascorbin®, 150 mg ascorbate/1 ml injection solution, pH7.0; Pascoe pharmazeutische Praeparate GmbH, Giessen, Germany), CoCl₂ and catalase (both from Sigma-Aldrich, Hamburg, Germany). Ascorbate or CoCl₂ was added directly to the culture medium of the cells. Cells treated with culture
Cells were treated with ascorbate at their respective IC₅₀ concentrations and incubated at 37°C for 24 hrs, which was considered significant. Two-way ANOVA was used for the statistical multiple comparisons of ascorbate-treated cells in combination with hypoxia and/or CoCl₂ treatment.

**Results**

Ascorbate generates ascorbyl and intracellular peroxide radicals in medium and in nine different cancer cell lines

We assessed the spontaneous generation of radicals from ascorbate in medium containing increasing percentage of foetal calf serum (FCS) by using ESR. The addition of ascorbate (8 mM) to the medium yielded a detectable amount of ascorbyl radicals (Asc•⁻; Fig. 1A). Increasing concentrations of FCS (0%, 1%, 10%, 40%, 99%) yielded increasing concentrations of ascorbyl radicals (Asc•⁻; Fig. 1A).
a correlating increase of detectable Asc$^-$ (Fig. 1A), suggesting that the formation of Asc$^-$ was catalysed by serum components, as previously reported by [3]. At pharmacological concentrations, ascorbate is a precursor of H$_2$O$_2$ generation in the extracellular milieu [7]. Therefore, the generation of H$_2$O$_2$ from increasing ascorbate concentrations in nine different cancer cell lines was measured. Addition of ascorbate (8 or 16 mM) resulted in an increase in H$_2$O$_2$ formation in all of the cancer cell lines (SF268, OVCAR-8, LOX IMVI, K562, NCI-H226, HT29, DU-145, HS-578T, SN12C) independent of the tumour type; as expected, the addition of 100 μg/ml catalase completely destroyed the ascorbate-induced H$_2$O$_2$ in all nine cell lines (Fig. 1B). Addition of H$_2$O$_2$ served as positive control (Fig. 1B).

### Ascorbate inhibits proliferation in all 60 cancer cell lines

We next analysed the cytotoxic efficacy of ascorbate on all cell lines of the NCI60 panel. To this end, the cells were incubated with 11 rising concentrations of ascorbate (31.25 μM–32 mM, double concentration in each step) to determine the cytostatic efficacy (inhibition of cell proliferation) for each cell line under normoxic conditions (21% O$_2$). The results of two exemplary cell lines (OVCAR-4 and NCI-H226) are displayed in Figure 2. In a second step, the exact IC$_{50}$ concentration was calculated. These two consecutive steps were performed for

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**Table 1:** Integrated signal of ascorbyl radicals in RPMI and RPMI supplemented with FCS.

| Sample          | Integrated signal Rel. ascorbyl |
|-----------------|---------------------------------|
| RPMI            | 0.00                            |
| RPMI, 0% FCS    | 3622.1                          |
| RPMI, 1% FCS    | 4143.1                          |
| RPMI, 10% FCS   | 4282.1                          |
| RPMI, 40% FCS   | 5032.1                          |
| RPMI, 99% FCS   | 9502.1                          |

**Table 2:** Integrated signal of ascorbyl radicals in RPMI and RPMI supplemented with FCS.

| Sample          | Integrated signal Rel. ascorbyl |
|-----------------|---------------------------------|
| RPMI            | 0.00                            |
| RPMI, 0% FCS    | 3622.1                          |
| RPMI, 1% FCS    | 4143.1                          |
| RPMI, 10% FCS   | 4282.1                          |
| RPMI, 40% FCS   | 5032.1                          |
| RPMI, 99% FCS   | 9502.1                          |

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**Fig. 1** Ascorbate generates ascorbyl and intracellular peroxide radicals in medium and in nine different cancer cell lines. (A) Induction of Asc$^-$ radicals from ascorbate in medium, measured by electron spin resonance (ESR) spectroscopy. Increasing serum concentrations yield more detectable Asc$^-$ radicals. All ESR measurements were performed in triplicates; shown is one representative image for each treatment group. (B) Nine different cancer cell lines were exposed to ascorbate (8 or 16 mM) with and without the addition of 100 μg/ml catalase in full medium. The generation of H$_2$O$_2$ after ascorbate treatment was measured in PBS by using dichlorofluorescein substrate. We detected an increase of H$_2$O$_2$ in all cell lines tested; this induction of H$_2$O$_2$ was completely blocked by catalase. Addition of 0.5 mM H$_2$O$_2$ to the cell cultures served as positive control. All H$_2$O$_2$ measurements were performed in quadruplicates; shown is mean ± SD. (A and B) RPMI: cell culture medium, FCS: foetal calf serum, asc.: ascorbate.
all 60 cell lines. We found a dose-dependent inhibition of cell proliferation in all cell lines, independent of their mutation status (Table S1) 24 hrs after treatment with pharmacological concentrations achievable in humans via i.v. administration of \(>10\) g ascorbate \([8]\) (Fig. 3A). Interestingly, susceptibility towards ascorbate-induced toxicity highly varied from the low micromolar range (leukaemia cell lines) to higher millimolar ranges \((e.g.,\) lung and prostate cancer cell lines; Fig. 3A). The average IC\(_{50}\) value of ascorbate for all 60 cancer cell lines under normoxic conditions was \(4.5 \pm 3.6\) mM. For the different tumour types, we found the following IC\(_{50}\) values of ascorbate:

- renal cancer cell lines \((n = 8)\): \(5.2 \pm 3.1\) mM;
- breast cancer cell lines \((n = 6)\): \(2.4 \pm 1.7\) mM;
- prostate cancer cell lines \((n = 2)\): \(13.2 \pm 11.1\) mM;
- colon cancer cell lines \((n = 7)\): \(3.7 \pm 3.8\) mM;
- lung cancer cell lines \((n = 9)\): \(5.9 \pm 3.6\) mM;
- leukaemia cell lines \((n = 6)\): \(0.6 \pm 0.7\) mM;
- melanoma cell lines \((n = 9)\): \(3.1 \pm 2.8\) mM;
- ovarian cancer cell lines \((n = 7)\): \(3.7 \pm 3.2\) mM;
- glioblastoma cell lines \((n = 6)\): \(3.0 \pm 1.3\) mM (Fig. 3A).

**Severe hypoxia significantly increases the IC\(_{50}\) concentrations of ascorbate in the 60 cancer cell lines**

Simultaneously to the determination of the IC\(_{50}\) value for ascorbate under normoxic conditions (21% O\(_2\)), the influence of severe hypoxia (0.1% O\(_2\)) on ascorbate-induced decrease in proliferation was analyzed. We chose 0.1% O\(_2\) treatment for 24 hrs to mimic chronic hypoxia as recently described for prostate cancer cells \([23]\). To this end, the different cell lines were cultured under hypoxic conditions during ascorbate exposure; hypoxia itself did not induce relevant apoptosis in the cell lines tested. We also detected a dose-dependent inhibition of cell proliferation in all 60 cancer cell lines; however, the IC\(_{50}\) value increased for every cell line (mean IC\(_{50}\) concentration under normoxia for all cell lines: \(4.5 \pm 3.6\) mM; mean IC\(_{50}\) concentration under hypoxia for all cell lines: \(10.1 \pm 5.9\) mM; \(P < 0.001\), Mann–Whitney \(U\)-test; Fig. 3B). This represented a global 2.2-fold increase in ascorbate IC\(_{50}\) value. In detail, we found the following IC\(_{50}\) values for ascorbate under hypoxic conditions (Fig. 3B; summarized in Table 1):

- renal cancer: 12.8 \pm 5.0 mM, 2.5-fold increase (five of eight cell lines showed a statistically significant increase of the IC\(_{50}\));
- breast cancer: 6.8 \pm 5.4 mM, 2.8-fold increase (five of six cell lines showed a statistically significant increase of the IC\(_{50}\));
- prostate cancer: 22.9 \pm 17.5 mM, 1.7-fold increase (one of two cell lines showed a statistically significant increase of the IC\(_{50}\));
- colon cancer: 8.6 \pm 5.6 mM, 2.3-fold increase (four of seven cell lines showed a statistically significant increase of the IC\(_{50}\));
- lung cancer: 12.2 \pm 8.0 mM, 2.1-fold increase (four of nine cell lines showed a statistically significant increase of the IC\(_{50}\));
- leukaemia: 1.2 \pm 1.3 mM, 2.0-fold increase (five of six cell lines showed a statistically significant increase of the IC\(_{50}\));
- melanoma: 9.7 \pm 9.7 mM, 3.1-fold increase (seven of nine cell lines showed a statistically significant increase of the IC\(_{50}\));
- ovarian cancer:
7.9 ± 7.5 mM, 2.1-fold increase (six of seven cell lines showed a statistically significant increase of the IC50); glioblastoma:
8.8 ± 0.8 mM, 2.9-fold increase (six of six cell lines showed a statistically significant increase of the IC50). The following cell lines did not reach a statistically significant increase of the respective IC50 under hypoxia: HT578T, HT-29, HCC2998, HCT-15, CCRF, NCI-H23, NCI-H226, M14, SK-MEL-5, OVCAR-8, PC-3, A498, RXF-393, ACHN.

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Hypoxia-induced ascorbate resistance is driven by HIF1α and by O2 partial pressure

To further determine whether the O2-dependent cytotoxic effects of ascorbate were dependent on the hypoxia-inducible factor-1α signaling cascade or simply on O2-availability, we applied CoCl2, a HIF1α-inducing drug [20] under normoxic and hypoxic conditions to allow the induction of HIF1α. Six cell lines of different tumour entities (TK10, HS578T, UACC257, HT29, SNB19, OVCAR8) that had shown a strong increase in the IC50 concentration of ascorbate under hypoxia (refer to Fig. 3B) were incubated with CoCl2 for 24 hrs under normoxic or hypoxic conditions. Western blot analyses with subsequent densitometry clearly demonstrated a strong induction of HIF1α upon CoCl2-treatment in five of the six cell lines (Fig. A and B). Hypoxia alone did not increase the level of detectable HIF1α, probably because of instability of the protein during protein extraction. Next, we analysed the influence of hypoxia alone, HIF1α (induced by CoCl2) or both on cancer cell viability after ascorbate exposure at increasing concentrations (0, 4, 8, and 16 mM) for 1 hr. Hypoxia rendered all of the cells resistant towards ascorbate-induced cell death (Fig. 4C). The same was true for CoCl2-treatment under normoxic conditions. Interestingly, the combination of both (hypoxia + CoCl2-treatment) significantly increased the ascorbate resistance in all of the six tested cancer cells. As expected, the addition of catalase blocked the ascorbate-induced proliferation inhibition (Fig. 4C).

High-dose ascorbate increases the sub-G1 cell population in the majority of the 60 cancer cell lines

To further determine whether the O2-dependent cytotoxic effects of ascorbate were conducted by apoptosis induction or via alterations in the cell cycle, fluorescence activated cell sorting (FACS) cell-cycle analyses were performed on all 60 cancer cell lines after incubation with ascorbate at the individual IC50 concentration for 24 hrs under normoxic (21% O2) and hypoxic (0.1% O2) conditions. The percentage of cells in the sub-G1 phase (indicating apoptotic cells) after ascorbate incubation varied between the different cell lines (Fig. 5 and Table S2), although the individual IC50 concentration of ascorbate determined in the proliferation assay (as described above) was applied in each case. Figure 5 depicts a representative cell line of each of the nine different tumour entities comprised in the NCI60 panel. Details of the cell-cycle distribution

under normoxic and hypoxic conditions after ascorbate treatment at the respective IC50 concentration of each of the 60 cancer cell lines are given in Table S2. Together, the FACS analyses demonstrate that pharmacological ascorbate primarily induced an increase in the sub-G1 cell population under normoxic conditions in 47 of 60 cells lines (Table S2). Under hypoxic conditions, we observed a comparable result after additional ascorbate treatment: In 45 of 60 cell lines, the sub-G1 cell population was increased. As expected (refer to Fig. 3), the sub-G1 cell population was decreased in the ascorbate + hypoxia group (in 38 of 60 cell lines) compared with the ascorbate + normoxia group.

Hypoxia and ascorbate treatment alters the expression of GLUT-1 in the 60 cancer cell lines

Finally, we tested whether the expression of the HIF-1α downstream-target GLUT-1 (responsible for the cellular dehydroascorbate uptake, competitively with glucose, [18]) was regulated by ascorbate in the 60 cell lines. Western blot analyses (and densitometry) were performed on the cell lysates 24 hrs after a 1 hr-exposure to ascorbate at the individual IC50 concentration under normoxic and hypoxic conditions. We could not detect a significant correlation between the level of endogenous GLUT-1 expression and susceptibility towards ascorbate-induced cell death (data not shown). However, our results yielded cell type-specific differences in GLUT-1 expression after ascorbate treatment and under hypoxia (Fig. 6). Under hypoxia (0.1% O2), the cellular GLUT-1 protein levels increased in 40 of the 60 cell lines. This effect occurred in most of the melanoma and breast cancer cells. This is in line with the fact that GLUT-1 is a downstream-target of HIF-1α, which is induced by hypoxia [24]. However, 10 of the 60 cell lines had a reduced GLUT-1 expression under hypoxic conditions (e.g. in the non-small cell lung cancer cell lines). Under normoxic conditions, ascorbate treatment at the individual IC50 concentrations decreased GLUT-1 expression in 30 of the 60 cell lines, with the most pronounced effects in melanoma, non-small cell lung cancer and breast cancer cell lines (Fig. 6), while in 22 of the 60 cell lines, an increased GLUT-1 expression was measured (most pronounced effect in ovarian and leukaemia cancer cell lines). In the last experimental setting (high-dose ascorbate + hypoxia), 20 cell lines had a reduced GLUT-1 expression (heterogeneous pattern; most pronounced in the breast cancer cell lines) and 31 cell lines an increased GLUT-1 expression (most pronounced effect in ovarian and colon cancer cell lines; Fig. 6).
Discussion

The clinical experience from phase I trials shows that in cancer patients, high-dose ascorbate bears only little cytotoxic efficacy [10, 19], which is in contrast to *in vitro* and *in vivo* pre-clinical data [3–6]. Cancer cells and the tumour microenvironment are hypoxic [13]. Therefore, in the current study, we asked whether hypoxia might be involved in the reduced efficacy of ascorbate in the killing of cancer cells. As analytical tool, we used the cell lines of the NCI60 panel for our experiments. In summary, our results clearly indicate that the generation of radicals from ascorbate was catalysed by serum and that severe hypoxia (0.1% O2) mediated a reduced susceptibility towards ascorbate-induced inhibition of cancer cell proliferation. This ascorbate resistance was enhanced by CoCl2-induced HIF1α. However, the ascorbate-induced cytotoxicity did not correlate with the endogenous GLUT-1 expression.

In a first approach, the individual IC50 values for ascorbate were determined for all 60 cell lines under normoxic (21% O2) and hypoxic (0.1% O2) conditions. Under normoxic conditions, all cell lines had IC50 values that were in the range of concentrations that can be achieved in patients by i.v. administration of ascorbate [4]. Hypoxia significantly increased the IC50 values. This might account for the fact that in pilot clinical trials on stage IV cancer patients by using high-dose pharmacological ascorbate, the cytotoxic efficacy was lower [10] than in defined cell culture experiments conducted under normoxic conditions [3]. Secondly, we asked whether the reduced susceptibility of the cancer cells towards ascorbate under hypoxic conditions was merely driven by the reduced O2 partial pressure (essential for radical formation) or whether HIF1α-signalling might also be involved in the observed cellular resistance. We used CoCl2 as inductor for HIF1α [22]. Our data show that both the decreased O2 partial pressure (hypoxia) and the expression of HIF1α drove resistance towards ascorbate-induced cell death. To determine the mode of

![Graphs showing the sub-G1 cell population under normoxic and hypoxic conditions](https://example.com/graphs)

**Fig. 5** Hypoxia and high-dose ascorbate increase the sub-G1 cell population in the 60 cancer cell lines. FACS cell-cycle analyses (100,000 cells measured per treatment group) were performed on all 60 cancer cell lines after incubation with or without ascorbate at the individual IC50 concentration for 24 hrs under normoxic (21% O2) and hypoxic (0.1% O2) conditions. Depicted is an exemplary cell line of each of the nine tumour entities of the NCI60 panel. The entire data are displayed in Table S2. Ascorbate treatment induced an increase in the sub-G1 cell population (apoptosis) under normoxic and hypoxic conditions. N: normoxia (21% O2), H: hypoxia (0.1% O2).
action of ascorbate-induced inhibition of cancer cell proliferation in more
detail, we performed FACS cell-cycle analyses on all 60 cell lines after
incubation with ascorbate at the individual IC50 under normoxic and hypoxic conditions. The FACS analyses yielded an increased sub-G1 (apoptotic) population in the majority of the cell lines after incubation with ascorbate under normoxic conditions, which was significantly reduced under hypoxic conditions. The high percentage of cells in the sub-G1 phase after incubation with ascorbate indicated that the induction of apoptosis was the major antiproliferative mode of action of ascorbate in cancer cells. However, in some of the cell lines analysed, no apoptosis induction was observed despite the obvious growth inhibition after incubation at the respective IC50 concentration. In such cases, pyknosis/necrosis probably caused the cytotoxic effects [3]. Finally, we analysed a possible role for GLUT-1 (which acts as pro-survival molecule in cancer cells [25]) in the susceptibility towards ascorbate-induce cell death. Western blot analyses demonstrated that hypoxic conditions increased the expression of the GLUT-1 protein in the majority of the 60 cell lines. Interestingly, ascorbate treatment decreased GLUT-1 expression under normoxic conditions in 30 of the cell lines, and under hypoxic conditions, ascorbate treatment reduced the pro-survival capacity for GLUT-1 up-regulation in nine of the 40 cell lines (from 40 to 31) and doubled the number of cell lines with a decreased expression of GLUT-1 under hypoxia alone (from 10 to 20), suggesting that high-dose ascorbate interferes with pro-survival mechanisms in the cancer cells. A limitation of the

![Fig. 6 Hypoxia and ascorbate treatment alter the expression of glucose transporter 1 (GLUT-1) in the 60 cancer cell lines. Western blot analyses were performed on the cell lysates of all 60 cell lines 24 hrs after exposure to ascorbate at the individual IC50 concentration for 1 hr under normoxic and hypoxic conditions. Protein expression was evaluated by densitometric analyses. Under hypoxia, the GLUT-1 protein level increased in 40 of the cell lines, most pronounced in melanoma and breast cancer cells. Ten cell lines had a reduced GLUT-1 expression under hypoxic conditions (e.g. in the non-small cell lung cancer cells). Under normoxia, ascorbate treatment reduced GLUT-1 expression in 30 cell lines, most pronounced in melanoma, non-small cell lung cancer and breast cancer cells; 22 cell lines showed an increased GLUT-1 expression, most pronounced in ovarian and leukaemia cancer cells. Under hypoxia + high-dose ascorbate, 20 cell lines had a reduced GLUT-1 expression, which was most pronounced in the breast cancer cells, while 31 cell lines had an increased GLUT-1 expression, most pronounced in ovarian and colon cancer cells.](image)
Western blot results on GLUT-1 expression is the impossibility of statistical quantification of the protein expression, as the Western blot experiments were only conducted once for each of the 60 cell lines at the four different experimental conditions, thus only allowing a qualitative analysis. As oxygenation (HBO treatment) of cancer patients does not increase tumour growth or the recurrence rate [16], our data imply that for future clinical application, cancer patients should be oxygenized before and during ascorbate infusions to maximize the cytotoxic efficacy.

Conclusion

Our results demonstrate that pharmacological doses of ascorbate bear cytotoxic effects on the NCI60 panel of cancer cells in vitro. This cytotoxicity is executed by ascorbyl radicals and H2O2 and is catalysed by serum components. Hypoxic conditions and HIF-1-ascorbate therapy to break drug resistance. Clinical trials, the respective patients should be oxygenized in addition to ascorbate in cancer patients up to this day. We conclude that in future clinical quantification of the protein expression, as the Western blot experiments were only conducted once for each of the 60 cell lines at the four different experimental conditions, thus only allowing a qualitative analysis. As oxygenation (HBO treatment) of cancer patients does not increase tumour growth or the recurrence rate [16], our data imply that for future clinical application, cancer patients should be oxygenized before and during ascorbate infusions to maximize the cytotoxic efficacy.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Source of the 60 cancer cell lines, and cell line-specific mutations.

Table S2 Cell-cycle FACS analyses of the 60 cancer cell lines und normoxia and hypoxia, with and without ascorbate treatment at the individual IC50 concentration.

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