H₂O₂-mediated Cytotoxicity of Pharmacologic Ascorbate Concentrations to Neuroblastoma Cells: Potential Role of Lactate and Ferritin

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Key Words

Pharmacologic Ascorbate • H₂O₂ • Ferritin • Lactate • Neuroblastoma • Warburg Effect

Abstract

By intravenous (but not oral) application of ascorbate, millimolar serum concentrations can be reached, which are preferentially cytotoxic to cancer cells. Cytotoxicity is mediated by transition metal-dependent generation of H₂O₂ in the interstitial space. In this study, the sensitivity of neuroblastoma cells (Kelly, SK-N-SH) to ascorbate and H₂O₂ and their defense mechanisms against H₂O₂ were investigated. Since aerobic glycolysis (the Warburg effect) is a feature of many tumour cells, their glucose consumption and lactate production were monitored. Furthermore, synthesis and release of ferritin by neuroblastoma cells were analysed in order to examine whether ferritin is possibly an iron source for H₂O₂ generation. Ascorbate (0.6–5.0 mM) and H₂O₂ (25–100 µM) were found to be similarly cytotoxic to Kelly and SK-N-SH cells. In each case, cytotoxicity increased if cell concentrations decreased, in accordance with low cell concentrations having lower capacities to detoxify H₂O₂. Kelly and SK-N-SH cells produced and released

remarkable amounts of lactate and ferritin. We propose the selective cytotoxicity of high dose ascorbate to tumour cells to be due to the preferential generation of H₂O₂ in the acidic and ferritin-rich tumour microenvironment, combined with reduced defense systems against H₂O₂ as a consequence of aerobic glycolysis.

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Introduction

There has been a long-lasting debate on the benefit of using ascorbate in cancer therapy. In the nineteen seventies, observational studies by Cameron and Pauling reported patients with terminal cancer to survive longer if treated with doses of 10 g ascorbate daily [1, 2]. This effect has not been found in two subsequent double-blind placebo-controlled studies at the Mayo Clinic, where 10 g ascorbate per day were administered orally [3, 4]. Currently, the use of ascorbate in cancer therapy is reinvestigated [5–7], considering that in Cameron's and Pauling's studies some patients received ascorbate also by intravenous (i.v.) application.

Since ascorbate serum levels achievable by oral and intravenous application differ dramatically [6, 8], data obtained from clinical trials need to be reinterpreted: Physiologic ascorbate serum concentrations are adjusted to about 30–60 μM by intestinal uptake, saturation of tissue transporters and renal excretion. After oral uptake of high ascorbate doses (1.5 g/day) the serum concentration increases up to 130 μM . Pharmacokinetic modeling predicts maximum levels of 220 μM after oral uptake of megadoses of 3 g ascorbate every 4 hours. In contrast, daily i.v.-administration of 1.5 g ascorbate achieves serum levels of up to 900 μM . For intravenous application of ascorbate doses clearly exceeding 1.5 g per day, serum peaks of up to 13 mM can be accomplished [8].

In the millimolar concentration range, ascorbate has been demonstrated to be cytotoxic to lung, breast, glioblastoma and various other cancer cell lines of murine, rat or human origin *in vitro*, in contrast to non-malignant cells [5, 7]. These effects have been shown to be mediated by the prooxidant properties of high dosed ascorbate. Its cytotoxicity is mediated by the generation of H_2O_2 in the interstitial space, which can easily penetrate cell membranes. Formation of H_2O_2 depends on the presence of transition metals such as iron usually safely bound to proteins [5, 7, 9].

These *in vitro* results have also been translated into promising effects in animal models. Growth rates of human ovarian carcinoma, murine pancreatic carcinoma and rat glioblastoma xenografts in mice have been significantly reduced by intravenous application of high doses of ascorbate [7]. In another study, growth rates of hepatocarcinoma xenografts in mice have also been shown to be inhibited by intravenous application of 1 g ascorbate per day per kg body weight, whereas oral application of the same dose has had no effect [10]. Considering the limited therapeutic options for many cancers and in light of the safety of intravenous ascorbate application [11], these data generate new interest in the use of ascorbate in pharmacologic concentrations for cancer therapy.

In this study we investigated the role of lactate and ferritin on the ascorbate induced anti-neuroblastoma activity mediated by H_2O_2 in Kelly and SK-N-SH cell lines. Both, lactate and ferritin, are increased in neuroblastoma; lactate is a result of glycolysis (Warburg effect) [12, 13] and ferritin is a well known tumour marker in neuroblastoma [14–16] which is released in significant amounts from the cells into the tumour microenvironment. The importance of these substances for the H_2O_2 -mediated cytotoxicity of ascorbate on cancer cells will be discussed in detail.

Materials and Methods

Materials

Ascorbic acid (ascorbate), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrogen peroxide (30% solution, H_2O_2), iron(II)sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium thiocyanate (KSCN), trichloroacetic acid (TCA) and Triton X-100 were purchased from Sigma (Taufkirchen, Germany). Fetal calf serum (FCS), L-glutamine, penicillin/streptomycin, Phosphate buffered saline without (PBS) and with Ca^{2+} and Mg^{2+} (PBS^{++}), RPMI 1640 medium and trypsin/EDTA were purchased from Biochrom (Berlin, Germany). Glucose oxidase (20 kU/68.5 mg lyo) and the test kits for the determination of D-glucose and L-lactate, respectively, were obtained from Roche (Grenzach-Wyhlen, Germany). The Delfia® Ferritin Kit for the determination of ferritin was from PerkinElmer (Rodgau-Jügesheim, Germany).

Cell lines and cell culture

The human neuroblastoma cell line Kelly was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). SK-N-SH was from ATCC (American Type Culture Collection, Maryland, USA). Cells were grown as monolayers in RPMI 1640 supplemented with 10% FCS (inactivated at 56°C for 30 min), L-glutamine (2 mM) and penicillin/streptomycin (100 U/ml/100 $\mu\text{g}/\text{ml}$). All cell cultures and cell suspensions both for cultivation of the cell lines and for experiments were maintained at 37°C in a 95% air/5% CO_2 atmosphere at 100% humidity. The medium was changed at intervals of 48 to 72 hours.

MTT-Test (proliferation and vitality assay)

Neuroblastoma cell cultures of various densities were grown by seeding various cell numbers of Kelly and SK-N-SH in 96-well plates (0.5×10^4 – 4×10^4 cells/200 μl medium/well) and maintaining them for 48 h. Subsequently, cell cultures were incubated with H_2O_2 (25–100 μM , f.c.) or ascorbate (0.6–5 mM, f.c.) for 48 h. Ascorbate was adjusted to pH 6.5–7.5 with NaOH. After incubation, cell viability was assessed by MTT assay according to Mosmann [17]: the supernatant was discarded, 100 μl RPMI 1640 containing MTT (0.5 mg MTT/ml) were added to each well and the plates were incubated for 3 h. After addition of 100 μl solvent (82% isopropanol, 9 % Triton X-100, 9 % 1 mM HCl; v:v) the solutions were homogenised for 12 h on a horizontal shaker in the dark. Absorbances were measured in a microplate reader at 570 nm wavelength and a reference wavelength of 630 nm. To establish calibration curves for MTT staining (cell number vs. absorbance) various cell suspensions were applied to 96-well plates: 2.5×10^4 – 1.5×10^4 Kelly cells/100 μl RPMI 1640/well or 2.5×10^4 – 1×10^4 SK-N-SH cells/100 μl RPMI/well. After addition of 10 μl MTT (0.5 mg MTT/ml RPMI 1640) the MTT assay was completed.

Generation and determination of H_2O_2

Detoxification of H_2O_2 by neuroblastoma cells: cell suspensions of Kelly or SK-N-SH were applied to 96-well plates (2×10^4 , 1×10^4 , 2×10^4 cells/200 μl RPMI 1640/well). These were

Fig. 1. Cytotoxicity of ascorbate (A) and H_2O_2 (B) to the human neuroblastoma cell line Kelly (upper part) and SK-N-SH (lower part). Correlation between cytotoxicity and cell number. Various numbers of Kelly or SK-N-SH cells were incubated with ascorbate (0.6–5 mM f.c.) or H_2O_2 (25–100 μ M f.c.) for 48 h. Viability was assessed by MTT assay. $n \geq 5$ for ascorbate and $n \geq 4$ for H_2O_2 , each performed 6-fold. Untreated controls set as 100%, mean $\pm$ SD.

then incubated either with 100 μ M H_2O_2 (f.c.) added as a bolus or with 1.4 mU glucose oxidase/200 μ l cell suspension. Glucose oxidase continuously generates H_2O_2 in catalysing the reaction: glucose (ca. 11 mM in RPMI 1640) + $O_2 \rightarrow$ gluconolactone + H_2O_2 . Incubation was stopped at intervals by adding 20 μ l TCA 50%.

H_2O_2 was analysed using the colorimetric method according to Thurman [18]: Samples were centrifuged (3 min, 15000 g). 50 μ l 10 mM $FeSO_4 \cdot 7H_2O$ (dissolved in 1 mM HCl) and 25 μ l 2.5 mM KSCN (dissolved in PBS⁺⁺) were added to 200 μ l of the supernatant. Absorbances were measured in a microplate-reader at 490 nm wavelength. Additionally calibration curves were assessed: for the staining of H_2O_2 in RPMI 1640, absorbances of RPMI 1640 containing various H_2O_2 -concentrations (0–200 μ M f.c.) and 20 μ l TCA (50%) were measured. For the production of H_2O_2 by glucose oxidase, 1.4 mU glucose oxidase were incubated in 200 μ l RPMI 1640. The reaction was stopped at intervals adding 20 μ l TCA 50% and H_2O_2 was stained.

Glucose consumption and lactate production in neuroblastoma cell cultures

50x10⁴ Kelly or SK-N-SH cells were seeded in 35 ml culture medium. After 48 h samples of 0.5 ml cell culture medium were taken ($t=0$) and replaced by 0.5 ml fresh culture medium. Samples were frozen at -20°C until glucose and lactate concentrations were determined. The procedure was repeated at intervals of 48 h. Glucose and lactate concentrations were measured by the test kits for the determination of glucose and lactate, respectively. Values were assessed according to the instructions of the provider.

Determination of ferritin

Cell suspensions of Kelly and SK-N-SH in PBS⁺⁺ (5x10⁶ cells/ml) were prepared. To determine the cellular ferritin content, 1.5 ml of the suspensions were centrifuged (5 min, 380 g) immediately after preparation ($t=0$). Cell pellets and supernatants were frozen at -80°C. To monitor the ferritin release, cell suspensions in PBS⁺⁺ were incubated in 6-well plates (1.5 ml/well) and, after 2, 4 and 10 hours, 200 μ l of each sample were taken. Samples were centrifuged (5 min, 380 g) and the supernatants were frozen at -80°C. Additionally, in a pilot experiment, release of ferritin from fibroblasts compared to SK-N-SH and Kelly cells (each 2.5 x 10⁶ cells/10 ml RPMI 1640) was measured after one, four and five days.

Ferritin contents were measured by a fluoroimmunoassay kit (Delfia® Ferritin Kit) with the detection based on a europium-labelled monoclonal antibody against human ferritin. Supernatants (20 μ l) were directly used for determination. Cell pellets were resuspended in 100 μ l demineralised H_2O refrozen on dry ice to enable cell disruption and centrifuged (3 min, 15000 g). Supernatants (20 μ l) were used for measurement. Ferritin contents were assessed following the instructions of the provider.

Results

Cytotoxicity of ascorbate and H_2O_2 to Kelly and SK-N-SH cells

The cytotoxicity of ascorbate and H_2O_2 to the human neuroblastoma cell lines Kelly and SK-N-SH was

Fig. 2. (A) Detoxification of an H_2O_2 -bolus (100 μM) by Kelly and SK-N-SH cells. Various cell numbers were incubated in RPMI 1640 containing 100 μM H_2O_2 ($t = 0$ min). (B) Detoxification of H_2O_2 continuously formed by glucose oxidase in the presence of Kelly and SK-N-SH cells. Various cell numbers were incubated in RPMI 1640 (ca 11 mM glucose) containing 1.4 mU glucose oxidase/200 μl cell suspension.

investigated with particular regard to its correlation with the cell concentration. Cell cultures of various cell numbers were incubated with ascorbate or H_2O_2 for 48 h. For ascorbate, concentrations between 0.6 mM (ca. triple the maximal plasma level after oral uptake) and 5 mM (easily achievable by i.v.-administration) were chosen (Fig. 1). H_2O_2 was used in micromolar concentrations between 25 μM and 100 μM , as more than 100 μM H_2O_2 have been detected after incubation of 5 mM f. c. ascorbate in cell culture medium (Fig. 1) [5].

Ascorbate and H_2O_2 were cytotoxic to Kelly and SK-N-SH cells and cytotoxicity increased with the substance concentration (Fig. 1). Through incubation of low concentrations of Kelly cells (4×10^4 cells) with 5 mM ascorbate or 100 μM H_2O_2 , nearly all cells were killed. For both substances and both cell lines, a reverse correlation between cytotoxicity and cell concentration was shown: the greater the number of Kelly or SK-N-SH cells incubated with ascorbate or H_2O_2 , the less vulnerable the cells were. This finding is consistent with the mediation of ascorbate cytotoxicity by H_2O_2 .

Detoxification of H_2O_2 by various cell concentrations

H_2O_2 easily diffuses through cell membranes and is detoxified by catalase and the GSH system in the intracellular space. With regard to the reverse correlation between cytotoxicity and cell concentration (Fig. 1), cellular capacities to detoxify H_2O_2 are expected to increase with cell concentration. To verify this hypothesis, various concentrations of Kelly and SK-N-SH cells (2×10^4 -

20×10^4 cells) were incubated with H_2O_2 , given as a bolus (100 μM), or with glucose oxidase simulating the continuous generation of H_2O_2 in the presence of ascorbate. The capacity of Kelly and SK-N-SH cells to detoxify H_2O_2 increased with the cell number and the incubation time, both if H_2O_2 was given as a bolus and if it was continuously formed (Fig. 2). Within 100 min, 100% of the H_2O_2 -bolus (100 μM) had been degraded by the 20×10^4 Kelly cells, with the 2×10^4 Kelly cells metabolising ca. 50% (Fig. 2A). Degradation of an H_2O_2 bolus by SK-N-SH was marginally less effective. After 120 min incubation of 2×10^4 and 20×10^4 cells, ca. 10% and 65% of the cells survived, respectively. Glucose oxidase (1.4 mU/200 μl RPMI 1640) continuously produced H_2O_2 in RPMI 1640 (containing ca. 11 mM glucose) generating an H_2O_2 concentration of ca. 150 μM f.c. within 40 min incubation time (Fig. 2B). Metabolisation of H_2O_2 , continuously formed by glucose oxidase, by Kelly and SK-N-SH cells showed similar efficiency. For both cell lines the H_2O_2 concentration of the cell culture medium amounted to ca. 80 μM H_2O_2 f.c. after 120 min incubation in presence of 20×10^4 cells and to ca. 200 μM H_2O_2 f.c. after 120 min incubation in presence of 2×10^4 cells.

Production and release of ferritin by neuroblastoma cells

It is assumed that transition metals such as iron are involved in the generation of H_2O_2 , where ascorbate is present [9, 10]. Various neuroblastoma cells are known to produce and release ferritin [14-16] which possibly serves as an iron source.

Fig. 3. (A): Release of ferritin by human neuroblastoma cells. Kelly and SK-N-SH cells (5×10^6 cells/ml) were incubated in PBS⁺⁺ and ferritin release into the supernatant was monitored for up to 10 h. $n=3$ for both cell lines, each performed in duplicate, mean $\pm$ SD. (B): Release of ferritin from Kelly and SK-N-SH cells in comparison to fibroblasts (each 2.5×10^6 cells/10 ml) after a 1, 4, and 5 days incubation period.

Fig. 4. Glucose consumption and lactate production in cell cultures of Kelly (A) and SK-N-SH (B). Glucose and lactate concentrations in cell culture medium were monitored for up to 144 h. First values were taken 48 h after seeding 50×10^4 cells in 35 ml RPMI 1640 ($t=0$).

This experiment investigated the ferritin content of Kelly and SK-N-SH cells and its release. Kelly cells, grown in RPMI 1640, contained 14.8 ± 8.1 ng ferritin/ 10^6 cells and SK-N-SH 7.76 ± 3.7 ng ferritin/ 10^6 cells ($n=3$). To monitor ferritin release, cells were incubated in PBS⁺⁺ (7.5×10^6 cells/1.5 ml) and ferritin levels in the supernatant were monitored for up to 10 h. Both cell lines were shown to discharge ferritin (Fig. 3A). After 10 hours, 21.8% and 6.4% of the total cellular ferritin content were detected in the supernatant of Kelly cells and SK-N-SH cells, respectively. Additionally, release of ferritin from fibroblasts in comparison to Kelly and SK-N-SH cells was followed for up to five days. As shown in Fig. 3B, the release from fibroblasts was definitely lower than from the malignant neuroblastoma cells.

Production and release of lactate by neuroblastoma cells

Many tumour cells produce and release elevated amounts of lactate using aerobic glycolysis (the Warburg effect) and thereby acidify their surroundings. Under acidic conditions, oxidation from Fe^{2+} to Fe^{3+} is less likely. Fe^{2+} is the biological active form and has a higher solubility than Fe^{3+} . Moreover, release of iron from binding proteins such as ferritin may be facilitated at low pH.

To investigate the glucose metabolism (rate of aerobic glycolysis) of neuroblastoma cells, glucose and lactate concentrations in the cell culture medium of Kelly

and SK-N-SH were monitored after seeding 50×10^4 cells in 35 ml cell culture medium. During an incubation period of 144 h, Kelly and SK-N-SH converted ca. 30% and ca. 41%, respectively, of the consumed glucose to lactate, causing high lactate concentrations in the supernatant (Kelly: ca 3.8 mM; SK-N-SH: ca 3 mM) (Fig. 4).

Discussion

The cytotoxicity of highly dosed ascorbate to tumour cells is mediated by the generation of H_2O_2 in the interstitial fluid. Millimolar ascorbate concentrations, which are required for this process, can only be attained by intravenous substance application. The selectivity of ascorbate cytotoxicity to malignant cells is not well-understood [5-7].

The present study verifies that millimolar ascorbate concentrations and H_2O_2 ($\sim 100 \mu\text{M}$) are cytotoxic to distinct neuroblastoma cell lines. There is a reverse correlation between the cytotoxicity and the cell concentration used. *In vitro*, samples with low cell concentrations, which can possibly be compared with small metastases *in vivo*, are destroyed most effectively. We propose that particularly two qualities of neuroblastoma (and some other malignant cells) are of importance for the preferred cytotoxicity of ascorbate: a) their high ferritin content and b) their high rate of aerobic glycolysis.

	low ferritin		high ferritin	
	0 mM AA	1 mM AA	0 mM AA	1 mM AA
DNA strand breaks	26%	89%	74%	90%
Poly (ADP-ribose) polymerase activity	340%	922%	396 %	500 %
NAD ⁺	68%	51%	27%	22%
ATP	96%	85%	58%	27%

Table 1. DNA strand breaks, increase of Poly (ADP-ribose) polymerase (PARP) activity and decrease of NAD⁺ and ATP concentrations in SK-N-SH cells after incubation with 100 μ M H₂O₂. Comparison of cells with low ferritin level (pretreated with 10 μ M DFO) and cells with high ferritin level (pretreated with 100 μ M FeSO₄) in the absence and presence of 1 mM ascorbate. Data are shown as percentage of controls (untreated SK-N-SH cells with low ferritin level and high ferritin level, respectively), controls were set as 0% for DNA strand breaks and set as 100% for PARP, NAD⁺ and ATP. Modified presentation of own data published earlier [27].

Ferritin as an iron source for the generation of reactive oxygen species (ROS)

Ascorbate can contribute to the formation of H₂O₂ only if transition metals such as iron (Fe²⁺) are present. The formation of H₂O₂ starts with the generation of superoxide (O₂^{•-}) [9, 19]. To initialise the generation of O₂^{•-}, minimal concentrations of “free” or “loosely bound” Fe²⁺ are necessary: Fe²⁺ + O₂ → Fe³⁺ + O₂^{•-}. Fe³⁺ can be re-reduced to Fe²⁺ by ascorbate. Accordingly, the production of remarkable amounts of O₂^{•-} can be sustained, if ascorbate abounds.

O₂^{•-} fulfils two important functions: The release of iron from ferritin and the spontaneous or SOD-catalysed disproportionation to H₂O₂ (and oxygen).

“Free” or “loosely bound” iron is highly cytotoxic. For this reason it is usually bound to proteins, for example to ferritin [14, 20], which can harbour up to 4500 iron ions (Fe³⁺) per complex [21]. Neuroblastoma contain elevated amounts of ferritin and release much higher amounts into the microenvironment than fibroblasts (Fig. 3). Fe²⁺ can effectively set free from ferritin by O₂^{•-} [19, 22]. So ferritin is of special interest as iron source for the generation of ROS via ascorbate. Fe²⁺ can also be released from ferritin by high concentrations of ascorbate, either directly or indirectly [23–26].

Besides its capacity to release iron from ferritin, O₂^{•-} is important for the generation of H₂O₂. In contrast to O₂^{•-}, H₂O₂ is a stable molecule and unrestrictedly penetrates cell membranes by diffusion. Within the cell, it can be detoxified by catalase or glutathione peroxidase (Fig. 2). If it is not effectively degraded, it indirectly causes oxidative damage such as DNA strand breaks [19]. The rate of H₂O₂-induced DNA strand breaks in SK-N-SH cells increases in the presence of ascorbate and elevated levels of ferritin. As a result, the activity of Poly (ADP-ribose) polymerase increases and the cellular NAD⁺ and

Fig. 5. Model for the generation of H₂O₂ in the tumour microenvironment, where ascorbate is present. Neuroblastoma cells are known to generate and release ferritin. Iron (Fe²⁺) can be set free from ferritin by O₂^{•-} and probably also directly by ascorbate. In the presence of O₂, Fe²⁺ is oxidized to Fe³⁺ and O₂^{•-} is formed. O₂^{•-} can disproportionate to H₂O₂ and O₂. The resulting Fe³⁺ can be re-reduced to Fe²⁺ by ascorbate [via ascorbylradical (AA•) formation] and the reaction is sustained. Many tumor cells acidify their microenvironment by aerobic glycolysis (release of lactate), which prevents formation of highly insoluble (i.e. inactive) Fe(OH)₃.

ATP pools are depleted, finally inducing cell death (Table 1 and Fig. 5) [27]. Similarly, peroxidation of lipids can be induced by treatment with ascorbate, if ferritin is present [28].

In addition to the extracellular processes proposed by Chen et al., ferritin/iron-dependent generation of ROS can also be triggered in the intracellular space: In cutaneous T-cell lymphoma cells, inhibition of ferritin synthesis by siRNA or NF- κ B-inhibition causes intracellular spill-over of “loosely fixed” iron, which results in apoptosis due to enhanced oxidative stress [29].

Even with iron and other transition metals kept safely,

considerable amounts of ROS accrue in every biological system, e.g. during oxygen consumption in the mitochondrial respiratory chain. As a consequence, cells dispose of well adapted defense mechanisms against ROS. Malignant cells, however, appear to have reduced capacities to cope with oxidative stress [19]. This feature is possibly associated with the high rate of aerobic glycolysis (the Warburg effect).

Aerobic glycolysis

Even under aerobic conditions, many tumour cells - such as neuroblastoma cells - convert remarkable amounts of glucose to lactate instead of using the oxidative phosphorylation, which is energetically much more effective (Warburg effect) [12, 13] (Fig. 4). Recently, several features of the Warburg effect could be clarified [30-33]. Regarding the H_2O_2 -mediated and iron-dependent cytotoxicity of ascorbate, two aspects may be of particular interest: First, the release of lactate: In a pilot experiment, release of lactate was found to be higher from neuroblastoma cells than from fibroblasts, leading to a lower pH in the microenvironment of the malignant cells (data not shown). Under acidic conditions, the formation of highly insoluble and inactive Fe^{3+} -precipitates [$Fe(OH)_3$] can rather be prevented and the release of iron from ferritin may be facilitated. Secondly, the generation of ROS is lower in cells of the Warburg type, as the rate of oxidative respiration is diminished. Consequently, cellular defense systems against oxidative stress are down-regulated and the cells become more sensitive to oxidative stress. The vulnerability of malignant cells by oxidative stress due to forced turn round of pyruvate to acetylCoA instead of the preferred LDH-dependent metabolism to lactate was demonstrated with different drugs such as dichloroacetate (inhibitor of pyruvate dehydrogenase(PDH)-kinase, α -lipoic acid (cofactor of

PDH), or FX11 or siRNA (inhibition of LDH A) [34-36]. Treatment with these substances increased endogenous generation of ROS in malignant cells and induced apoptosis. In analogy, increased generation of ROS (H_2O_2) can also be take place outside the cells by ferritin/ascorbate.

In conclusion: The confrontation of cancer cells with oxidative stress is a reasonable concept for cancer therapy. During the last years, the pro-oxidative cytotoxic effects of high ascorbate concentrations to cancer cells have been investigated in detail, both in the cell culture and in the animal model. The serum ascorbate concentrations, which are necessary to induce such effects, can easily be attained in humans by intravenous substance application [37, 38]. Compared to the majority of aggressive chemotherapeutic agents, ascorbate may only have moderate anti-cancer effects, but almost no side effects [39, 40]. Particularly this quality fits well into a novel concept for cancer therapy, which is being considered with regard to the poor profit and the severe side effects of many current chemotherapies. It intends "to design therapies to sustain a stable tumour mass rather than eradicate all cancer cells" [41, 42]. As ascorbate certainly is a promising substance for such an approach and the intravenous application of high substance doses is excellently tolerated by humans, high-dosed ascorbate should be introduced into the therapy of neuroblastoma and other malignancies.

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