Free Radical Biology & Medicine 47 (2009) 32–40

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects

Julien Verrax, Pedro Buc Calderon *

PMNT Unit, Toxicology and Cancer Biology Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, 1200 Bruxelles, Belgium

ARTICLE INFO

Article history:
Received 2 October 2008
Revised 11 February 2009
Accepted 12 February 2009
Available online 28 February 2009

Keywords:
Ascorbate
Vitamin C
Oxidative stress
Cancer
Chemotherapy
Free radicals

ABSTRACT

Recently, it has been proposed that pharmacologic concentrations of ascorbate (vitamin C) can be reached by intravenous injection. Because high doses of ascorbate have been described to possess anticancer effects, the therapeutic potential of these concentrations has been studied, both in vitro and in vivo. By using 2-h exposures, a protocol that mimics a parenteral use, we observed that pharmacologic concentrations of ascorbate killed various cancer cell lines very efficiently (EC_{50} ranging from 3 to 7 mM). The mechanism of cytotoxicity is based on the production of extracellular hydrogen peroxide and involves intracellular transition metals. In agreement with what has been previously published, our in vivo results show that both intravenous and intraperitoneal administration of ascorbate induced pharmacologic concentrations (up to 20 mM) in blood. In contrast, the concentrations reached orally remained physiological. According to pharmacokinetic data, parenteral administration of ascorbate decreased the growth rate of a murine hepatoma, whereas oral administration of the same dosage did not. We also report that pharmacologic concentrations of ascorbate did not interfere with but rather reinforced the activity of five important chemotherapeutic drugs. Taken together, these results confirm that oral and parenteral administration of ascorbate are not comparable, the latter resulting in pharmacologic concentrations of ascorbate that exhibit interesting anticancer properties.

Vitamin C (ascorbic acid) has a controversial history in cancer therapy. Thirty years ago, the Nobel Prize winner Linus Pauling and the Scottish physician Ewan Cameron published two retrospective studies reporting the prolongation of survival times in terminal human cancer by the administration of high doses of vitamin C [1,2]. Their rationale was that ascorbate might promote host resistance in advanced cancer patients [3], who generally present low concentrations of ascorbic acid in plasma [4,5]. However, this fact is currently known to be mainly correlated with the low dietary intake of vitamin C presented by these patients [6,7] or is sometimes the consequence of the chemotherapeutic treatment [8]. As Cameron and Pauling's studies did not follow the standard rules of clinical trials, their conclusions were soon afterward refuted by different prospective, controlled, and double-blind studies showing that there was no difference in the survival of patients receiving oral vitamin C and those receiving a placebo [9,10]. Many years later, it has been remarked that the protocols used in the latter studies were slightly different [11,12]. The treated group of Cameron and Pauling consisted of patients who were taking 10 g of ascorbate per day, first intravenously for about 10 days and then orally. In contrast, the double-blind studies also used 10 g per day, but only orally. This fact is probably not the only element that explains differences between the results of the studies, but it could have a critical importance given the particular pharmacokinetics of ascorbic acid. Indeed, it was nicely shown in humans that blood concentrations of ascorbate are tightly controlled as a function of oral dose [13,14]. As a consequence, complete plasma saturation occurs at oral doses of ≥400 mg daily, achieving physiological blood concentrations of 60–100 μM. In contrast, intravenous infusions of ascorbate have been reported to achieve plasma concentrations up to 20 mM, which is 200 times more than what it is possible to reach orally [12].

Interestingly, at this range of pharmacologic concentrations (0.3–20 mM), ascorbate exhibits a strong cytolytic activity in vitro against a wide variety of cancer cells [15–17], which seem to be strikingly more sensitive than normal cells [18]. Because we had previously used ascorbate to potentiate the cytotoxic effects of redox-active compounds [19,20], we decided to study its own anticancer properties. We investigated the pharmacokinetics of ascorbate in mice, considering

Abbreviations: ROS, reactive oxygen species; NAC, *N*-acetylcysteine; CAT, catalase; TLT, transplantable liver tumor; PBS, phosphate-buffered saline; C-DCHF-DA, carboxydichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; ip, intraperitoneal; iv, intravenous; EDTA, ethylenediaminetetraacetic acid; DFO, deferoxamine mesylate; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; UV, ultraviolet; ANOVA, analysis of variance; AUC, area under the curve.

* Corresponding author. Fax: +32 2 764 73 59.
E-mail address: pedro.buccalderon@uclouvain.be (P.B. Calderon).

0891-5849/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2009.02.016

different routes of administration: oral, intravenous (iv), and intraperitoneal (ip). We then investigated the cytolytic activity of ascorbate in vitro, at various concentrations, against various human cancer cell lines. Cells were incubated with ascorbate for only 2 h to mimic a potential clinical iv use. Finally, the antitumoral activity of both oral and parenteral administration of ascorbate was investigated in a tumor-bearing mouse model.

Our in vitro results confirm that pharmacologic concentrations of ascorbate are cytotoxic for cancer cells. The mechanism of cytotoxicity is likely based on the production of reactive oxygen species (ROS), because *N*-acetylcysteine and catalase, two powerful antioxidants, were both able to completely suppress cell death. The cytolytic process involves intracellular reactive metals given that preincubation of cells with a cell-permeant chelator prevented cell death. In vivo, parenteral administration of ascorbate diminished the growth of a murine hepatoma, whereas oral administration of the same dosage (1 g/kg) did not. This is in agreement with pharmacokinetic data, which show that pharmacologic concentrations of ascorbate cannot be achieved orally. Finally, ascorbate is reported to reinforce the efficacy of five chemotherapeutic drugs possessing different mechanisms of action. Taken together, our data confirm previous results showing that the effects of oral and parenteral administration of ascorbate are not comparable [12]. They also confirm that pharmacologic concentrations of ascorbate possess interesting anticancer properties [18,21].

Materials and methods

Cell lines

The murine hepatoma cell line "transplantable liver tumor" (TLT) was cultured in Williams' E essential medium supplemented with 10% fetal calf serum, glutamine (2.4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml). The cultures were maintained at a density of $1–2\times10^5$ cells/ml and the medium was changed at 48- to 72-h intervals. Human cancer cell lines (T24, DU145, MCF7, HepG2, Ishikawa) were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere at 100% humidity. Phosphate-buffered saline (PBS) was purchased from Gibco.

Chemicals

Sodium ascorbate (vitamin C), *N*-acetylcysteine (NAC), carboxydichlorodihydrofluorescein diacetate (C-DCHF-DA), sanguinarine, ethylenediaminetetraacetic acid (EDTA), deferoxamine mesylate (DFO), diethylenetriaminepentaacetic acid (DTPA), protease inhibitor cocktail, catalase, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). The various chemotherapeutic agents (etoposide, paclitaxel, 5-fluorouracil, cisplatin, and doxorubicin) were also purchased from Sigma. Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals were ACS reagent grade.

Cell survival assays

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (10,000 per well) were plated in a 96-well plate and allowed to attach overnight. Forty-eight hours after treatment, cells were washed two times with warm PBS, and MTT-containing medium was added to the wells for 2 h at 37°C. At the end of the incubation, the supernatant was discarded, 100 μl of DMSO was added, and the absorbance was then measured at 550 nm in a microtiter plate reader. For suspension-growing cells (TLT), the viability was estimated by measuring the activity of lactate dehydrogenase (LDH) according to the procedure of Wroblewski and Ladue [22], both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as the ratio of released activity to the total activity.

Clonogenic assays were performed by seeding cells (500) in six-well plates at a single-cell density and allowing adherence overnight. They were then treated with ascorbate for 2 h, washed with warm PBS, given fresh medium, and allowed to grow for 10 days. Clonogenic survival was determined by staining colonies using crystal violet.

Measurement of ATP content

ATP content was determined by using the Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) according to the procedures described by the supplier.

Measurement of glutathione content

The content of reduced glutathione was determined by using the GSH-Glo glutathione assay (Promega, Madison, WI, USA) according to the procedures described by the supplier.

Assessment of ROS formation

C-DCHF-DA was used to detect ROS production. Cells were grown in Lab-Tek chamber plates and incubated either in the presence or in the absence of ascorbate for 30 min. They were then incubated at 37°C for 20 min in 1 ml of 1 μM C-DCHF-DA and visualized under a fluorescence microscope from Optika (Ponteranica, Italy). Pictures were taken with a Moticam 2300 from Motic (Hong Kong, China).

Annexin-V/propidium iodide staining

Cells were harvested at different times of incubation and stained with the Roche Annexin-V–Fluos Staining Kit following the manufacturer's instructions. Cells were then observed under a fluorescence microscope, as previously described.

Animals

Six-week-old female NMRI mice were used for all in vivo studies. Tumor implantation was done by injecting 10^6 syngeneic TLT hepatocarcinoma cells into the gastrocnemius muscle in the right hind limb of the mice, at the vicinity of the great saphenous vein. Tumor diameters were tracked three times a week with a caliper and tumor volumes were calculated according to the following formula: $(\text{length}\times\text{width}^2\times\pi)/6$. Each procedure was approved by the local authorities according to national animal care regulations.

Ascorbate administration

Ascorbate-treated groups were either injected ip or received a bolus dose of 1 g/kg of sodium ascorbate, according to the described schedules. Ascorbate solutions were prepared daily in injectable water for ip and iv injections. For iv injections, animals were anesthetized with sodium pentobarbital (Nembutal; 60 mg/kg weight) before a single injection into the tail vein. The ascorbate solutions were hypertonic, compared to control mice, which were injected with sodium chloride 0.9% only. Water for injections and sodium chloride 0.9% were both purchased from B Braun (Melsungen, Germany). For the oral supplementation, ascorbate was added to the drinking water at 6 g/L. Based on a daily water consumption of ~5 ml per day, this corresponds to a daily administration of 1 g/kg, the same dose as was used for parenteral injections.

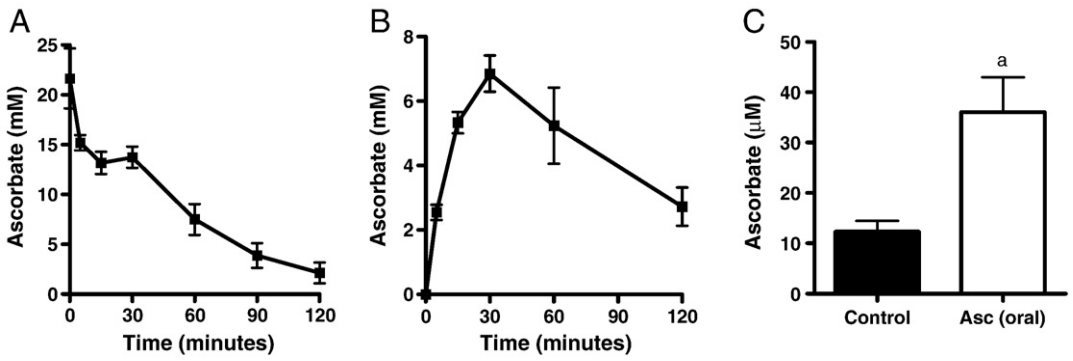

Fig. 1. Pharmacologic concentrations of ascorbate are achieved by either iv or ip injection. (A) Pharmacokinetic profile obtained after the iv administration (tail vein) of 1 g/kg of ascorbate in mice. Data were obtained from 6 animals. Blood samples were taken at various times (5, 15, 30, 60, 90, and 120 min), and plasma was isolated and stored at −80°C, as described under Materials and methods. The ascorbate levels were quantified within the week using HPLC coupled with UV detection. Pharmacokinetic data were analyzed using a noncompartmental analysis. (B) Pharmacokinetic profile obtained after the ip administration of 1 g/kg of ascorbate in mice. Data were obtained from 6 animals. (C) Mean plasma concentrations of ascorbate were determined from 12 and 6 animals, for control and ascorbate-treated group, respectively. Ascorbate was added to the drinking water for 4 weeks at 6 g/L, as described underMaterial and methods. [a] $p<0.001$ versus control.

Blood sampling

Blood samples were obtained from the lateral saphenous vein and collected in Eppendorf vials containing tripotassium EDTA as anticoagulant. They were kept on ice before being centrifuged at 2000*g* at room temperature for 5 min to obtain plasma. Plasma samples were then stored at −80°C and analyzed within 1 week.

Ascorbate quantification

Plasma samples were deproteinized by adding half a volume of a solution containing 20% metaphosphoric acid and 6 mM EDTA. They were then centrifuged at 13,000*g* at 4°C for 10 min. Supernatants were collected and immediately processed. Ascorbate levels were measured by reverse-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, according to a protocol adapted from Emadi-Konjin et al. [23]. The HPLC apparatus consisted of a Kontron 420 pump (Kontron Instruments, Eching, Germany) equipped with a Waters 2487 dual-wavelength absorbance detector (Waters Associates, Milford, MA, USA). Samples were transferred to the column by a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, using a glass 100-mm^3 Hamilton syringe (Hamilton, Reno, NV, USA). Separations were achieved on a Nucleosil C18 column (Grace Davison Discovery Sciences, Deerfield, IL, USA) equipped with an All-Guard C18 precolumn (Alltech, Breda, The Netherlands). The mobile phase contained 2 mM EDTA and consisted of 0.2 M KH_2PO_4 adjusted to pH 3 with H_3PO_4. The UV detector was set at 254 nm and the flow rate was 1 ml/min. Ascorbate standards (0–50 μM) were used to provide a calibration curve. They were prepared daily and treated in the same way as plasma samples.

Analysis of pharmacokinetic data

GraphPad Prism software was used for all calculations (GraphPad Software, San Diego, CA, USA). EC_{50} values were determined by nonlinear regression. Pharmacokinetic data were analyzed by using a noncompartmental analysis and the following parameters were determined (when required): the initial peak concentration ($C^\circ p$), peak concentration in plasma (C_{max}), time to C_{max} (T_{max}), area under the curve (AUC; $0 \rightarrow \infty$), clearance (CL), fraction of drug absorbed (bioavailability) (F), and elimination half-life ($t_{1/2}$). Both the $C^\circ p$ and the elimination rate constant (k) were estimated by using linear regression performed on the logarithm of plasma concentrations. The $t_{1/2}$ was calculated using the equation $t_{1/2} = \ln 2/k$. The AUC values (up to 120 min) were calculated using GraphPad Prism, and the extrapolation to infinity was calculated by dividing the last measured concentration by k. The CL was calculated by dividing the dose by the AUC (multiplied by the bioavailability in the case of ip administration). The F for ascorbate after ip injection was calculated by dividing the AUC after ip administration by the AUC after iv administration.

Statistical analysis

Results are presented as mean values and the error bars represent the standard error of the mean. Data were analyzed by using one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test for significant differences between means. For statistical comparison of results at a given time point, data were analyzed using Student's t test.

Results

Pharmacologic concentrations of ascorbate are achieved by either iv or ip injection

First of all, the pharmacokinetics of ascorbate were investigated. Pharmacokinetic profiles were obtained from six mice receiving either iv (as a bolus) or ip 1 g/kg of sodium ascorbate, a dose that is similar to the pharmacologic doses used in humans [24,25]. The intravenous injection achieved plasmatic concentrations in the millimolar range, as soon as 5 min after the injection (Fig. 1A). From the noncompartmental analysis, the $C^\circ p$ was estimated to be 22 ± 3 mM (Table 1). Intraperitoneal injections resulted in a peak plasma concentration of 7 ± 1 mM, 30 min after injection (Fig. 1B). The average basal ascorbate concentration in these animals was only 12 ± 3 μM; this means that the parenteral administration of ascorbate achieves pharmacologic concentrations in blood that are approximately 500–2000 times higher than physiological concentrations. A relatively short half-life was found for both routes of administration, 40 ± 8 and 54 ± 9 min, for iv and ip, respectively. Ascorbate levels returned to normal values (19 ± 2 μM, $n = 4$) 24 h after an ip injection,

Table 1
Pharmacokinetic data of parenteral administration of ascorbate

Parameter	ip	iv
$C^\circ p$	–	22 ± 3 mM
T_{max}	0.5 h	–
C_{max}	7 ± 1 mM	–
$t_{1/2}$	54 ± 9 min	40 ± 8 min
CL	9 ± 2 ml/h	8 ± 1 ml/h
AUC $0 \rightarrow \infty$	12 ± 3 mM h	20 ± 2 mM h
F	0.62	–

suggesting that no accumulation occurs. From the respective AUC values, the bioavailability (F) for ip administration was estimated to 0.62 (Table 1).

The effect of oral supplementation was also investigated in a group of six animals treated with 1 g/kg of ascorbate in the drinking water for 4 weeks. At the end of the treatment, a significant threefold increase in ascorbate concentration was found (Fig. 1C), consistent with previously published results in rats [26]. However, plasma ascorbate concentrations after oral administrations remained below 50 μM, which is more than 100 times less than what can be reached parenterally. Similar findings have been described in humans [13,14] and confirm that pharmacologic concentrations cannot be reached orally.

Various cancer cell lines are killed by pharmacologic concentrations of ascorbate

Because pharmacologic concentrations of ascorbate can be reached in vivo, we then assessed the anticancer activity of such concentrations in vitro. For that purpose, several human cancer cell lines representing different cancer types were used: T24 (bladder), DU145 (prostate), HepG2 (liver), MCF7 (breast), and Ishikawa (cervix). They were incubated in the presence of various concentrations of ascorbate, from 50 μM, which corresponds to baseline plasma vitamin C concentrations observed in humans [27,28], up to 33 mM (Fig. 2A). A 2-h exposure was used for all the in vitro experiments, to mimic a clinical iv use, and cellular viability was checked 48 h later using the MTT reduction assay. Similar profiles of toxicity were observed in all cell lines tested and the EC_{50} ranged from 3 to 7 mM. Interestingly,

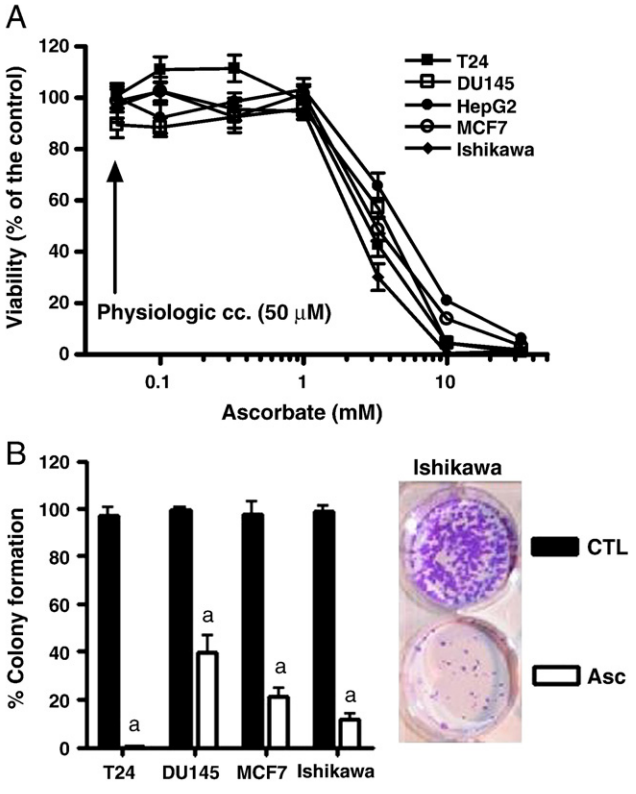

Fig. 2. Various cancer cell lines are killed by pharmacologic concentrations of ascorbate. (A) Cells (10,000) were treated with various concentrations of ascorbate for 2 h, washed twice with PBS, and reincubated in fresh medium. Viability was assessed by MTT 48 h after the exposure to ascorbate, as described under Materials and methods. Results are means of three separate experiments performed in triplicate. (B) Cells (500) were treated with 5 mM ascorbate for 2 h, then washed twice with PBS, and reincubated in fresh medium for 10 days. Colonies were then visualized by using crystal violet. Results are means of two separate experiments ($n=5$). $^{a}p<0.001$ versus control.

Table 2
EC_{50} of sodium ascorbate on various human cancer cell lines

Cell line	EC_{50} (mM)	Apoptotic defect
T24	3.4	Mutation in p53
DU145	5.8	Mutation in p53
HepG2	7.1	Wild type
MCF7	4.5	Mutation in caspase-3
Ishikawa	2.9	Wild type

these values were related to neither p53 nor caspase-3 cell status (Table 2), suggesting that defects in the apoptotic pathway do not influence ascorbate toxicity. It should be noted that these cytotoxic concentrations can be easily reached by parenteral administration (especially iv), which allows, for at least 1 h, pharmacologic concentrations above any EC_{50}. The toxicity of pharmacologic concentrations of ascorbate was further confirmed in a clonal survival assay in which we observed that a 2-h exposure to 5 mM ascorbate led to a decrease of 61 to 99% in the number of colonies (Fig. 2B).

Pharmacologic concentrations of ascorbate generate extracellular hydrogen peroxide, which reacts with intracellular metals

Because the formation of hydrogen peroxide (H_2O_2) by pharmacologic doses of ascorbate was described both in vitro and in vivo [18,29], we assessed the putative role of ROS in the cytotoxic process. We observed that NAC and catalase (CAT), two H_2O_2 scavengers, completely suppressed cell death in all cell lines tested (Fig. 3A). It should be emphasized that catalase also had a protective effect in the clonal survival assay, whereas heat-inactivated catalase was ineffective (data not shown). Because catalase is likely membrane impermeative, such an observation confirms that formation of hydrogen peroxide occurs extracellularly [18,29].

As the presence of reactive oxygen species seemed paradoxical given the well-known antioxidant properties of ascorbate, we decided to use a fluorescent ROS-sensitive probe, namely C-DCHF-DA. Our results show that an increase in intracellular ROS can be detected as soon as 30 min after the exposure of cells to ascorbate (Fig. 3B). Supporting the occurrence of an oxidative stress, we observed decreases of respectively 20 and 40% in GSH and ATP 4 h after the exposure to ascorbate, in all the cell lines tested (T24, DU145, MCF7, and Ishikawa) (data not shown). The addition of various chelators suggests that intracellular metals participate in ascorbate toxicity. Indeed, preincubation with DFO, a cell-permeant metal chelator, prevented the loss of viability of tumor cells exposed to pharmacologic concentration of ascorbate (Fig. 3C). In contrast, two cell-impermeant chelators, namely EDTA and DTPA failed to protect against ascorbate toxicity (Fig. 3D). Confirming the importance of intracellular metals in ascorbate toxicity, we observed that DFO also had a protective effect in the clonal survival assay (Fig. 3E).

Cancer cells exposed to pharmacologic concentrations of ascorbate die through a necrotic cell death

Regarding the type of cell death induced by pharmacologic concentrations of ascorbate, we observed that a broad caspase inhibitor did not protect against cell death (Fig. 4A). This result suggests that caspases are not involved in the cytolytic process, a fact that was further confirmed by the absence of any DEVDase activity in ascorbate-treated cells (data not shown). We then looked for cell membrane integrity and phosphatidylserine translocation using a double annexin-V and propidium iodide staining. As shown in Fig. 4B, ascorbate-treated cells exhibit a clear necrotic profile (annexin-V and propidium iodide positive), as soon as 8 h after the exposure to ascorbate. Overall, these results confirm the observation made by Chen et al. that, in vitro, necrosis is the main type of cell death induced by pharmacologic concentrations of ascorbate [18].

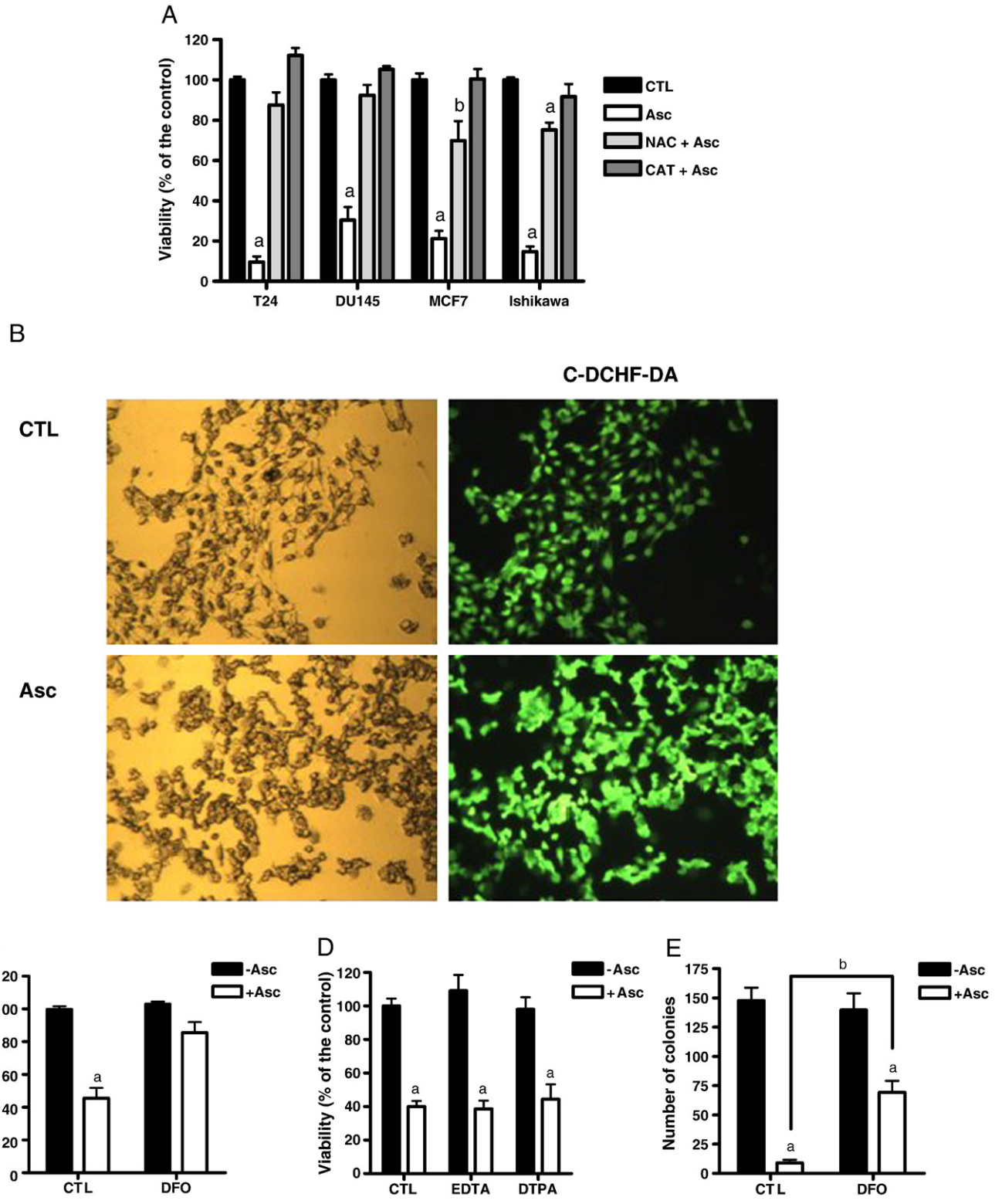

Fig. 3. Pharmacologic concentrations of ascorbate generate extracellular hydrogen peroxide, which reacts with intracellular metals. (A) Cells (10,000) were treated with various concentrations of ascorbate for 2 h either in the presence or in the absence of inhibitors. They were then washed twice with PBS and reincubated in fresh medium. Viability was assessed by MTT assay 48 h after the exposure to ascorbate, as described underMaterial and methods. Results are means of three separate experiments performed in triplicate. $^{a}p<0.001$ and $^{b}p<0.01$ versus control. (B) T24 cells (10,000) were seeded in Lab-Tek chambers as described under Materials and methods. They were then treated with 10 mM ascorbate for 30 min, washed, and incubated for a further 20 min in the presence of 1 μM C-DCHF-DA. (C and D) DU145 cells (10,000) were first preincubated for 3 h with different chelators used at the following concentrations: EDTA, 1 mM; DFO, 500 μM; DTPA, 1 mM. They were then washed twice with PBS and reincubated in fresh medium containing 10 mM ascorbate for 2 h (either in the presence or in the absence of chelator for the results shown in D). At the end of the incubation, cells were washed twice with PBS and reincubated in fresh medium. Viability was assessed by MTT assay, 48 h after the exposure to ascorbate, as described under Materials and methods. Results are means of three separate experiments performed in quadruplicate. $^{a}p<0.001$ versus control. (E) DU145 cells (500) were first preincubated for 3 h in the absence or in the presence of DFO (500 μM). They were then washed twice with PBS and reincubated in fresh medium containing 10 mM ascorbate for 2 h. At the end of the incubation, the cells were washed twice with PBS and reincubated in fresh medium for 10 days. Colonies were visualized by using crystal violet. Results are means of two separate experiments performed in triplicate. $^{a}p<0.001$ and $^{b}p<0.01$ versus control.

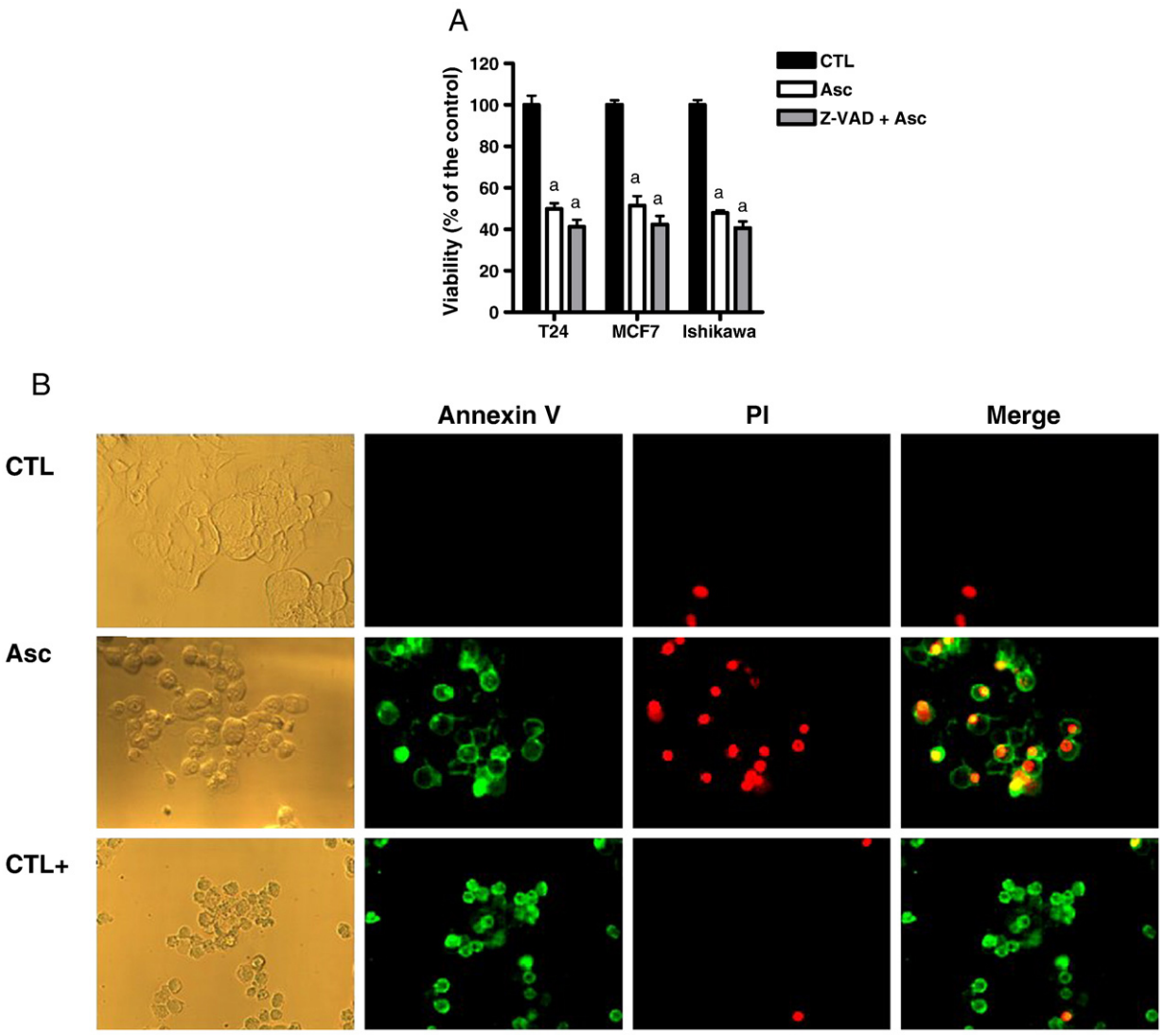

Fig. 4. Cancer cells exposed to pharmacologic concentrations of ascorbate die through a necrotic cell death. (A) Cells (10,000) were exposed to 10 mM ascorbate for 2 h, either in the absence or in the presence of a broad caspase inhibitor (Z-VAD-fmk, 50 μM), which was preincubated for 1 h. They were then washed twice with PBS and reincubated in fresh medium in the absence or in the presence of the caspase inhibitor. Viability was assessed by MTT assay 48 h after the exposure to ascorbate, as described under Materials and methods. Results are means of three separate experiments performed in triplicate. $^{a}p<0.001$ versus control. (B) T24 cells (10,000) were seeded in Lab-Tek chambers, allowed to attach, treated with 10 mM ascorbate for 2 h, washed, and incubated for a further 6 h. Sanguinarine (10 μM) was used as a positive control for apoptosis, as previously described [19]. Double staining with annexin-V/propidium iodide was performed as described under Materials and methods.

Parenteral administration of ascorbate decreases tumor growth rate

Because pharmacologic ascorbate concentrations can be reached in vivo, and as they induce cell death in various cancer cell lines in vitro, we looked for a putative anticancer effect in a tumor-bearing mouse model. For that purpose, we chose the murine TLT cell line [30], which allows a solid tumor growth when implanted in mice [31–34]. We first assessed the in vitro sensitivity of these cells toward ascorbate. As observed for other cell lines, TLT cells were efficiently killed by pharmacologic concentrations of ascorbate and presented an EC_{50} value of approximately 6 mM (Fig. 5A). Our results show that a daily administration of ascorbate (1 g/kg ip) significantly decreased tumor growth rate (Fig. 5B). At the end of the treatment, mean tumor volume in the control group reached 2200 ± 300 mm^3 versus 1300 ± 200 mm^3 for the ascorbate-treated group, which means a significant decrease of 40% ($p<0.01$) (Fig. 5C). Interestingly, no sign of any side effect was observed (no decrease in body weight, no abnormal behavior) despite repeated injections of pharmacologic doses of ascorbate. As shown in Fig. 5D, the oral supplementation of ascorbate in drinking water failed to induce any decrease in final tumor volumes (2400 ± 400 cm^3 versus 2200 ± 200 cm^3, for control and ascorbate-treated mice, respectively). This result is in agreement with our pharmacokinetic data, which suggest that pharmacologic concentrations of ascorbate cannot be reached orally.

Pharmacologic concentrations of ascorbate do not inhibit the activity of chemotherapeutic agents but rather reinforce their cytolytic effect

Because parenteral administration of pharmacologic doses of ascorbate was reported to be safe [25], one interesting possibility might be to administer this compound in combination with other cytotoxic agents. We therefore assessed a putative interference of ascorbate with five chemotherapeutic drugs representing the main existing classes: etoposide (nonintercalating topoisomerase-targeting drug), cisplatin (alkylating agent), 5-fluorouracil (antimetabolite), doxorubicin (intercalating topoisomerase-targeting drug), and paclitaxel (microtubule-targeting drug). These drugs were used at concentrations representing their respective EC_{50} values, either in the absence or in the presence of ascorbate, for which two concentrations were assessed: 50 μM and the EC_{50} value. As shown

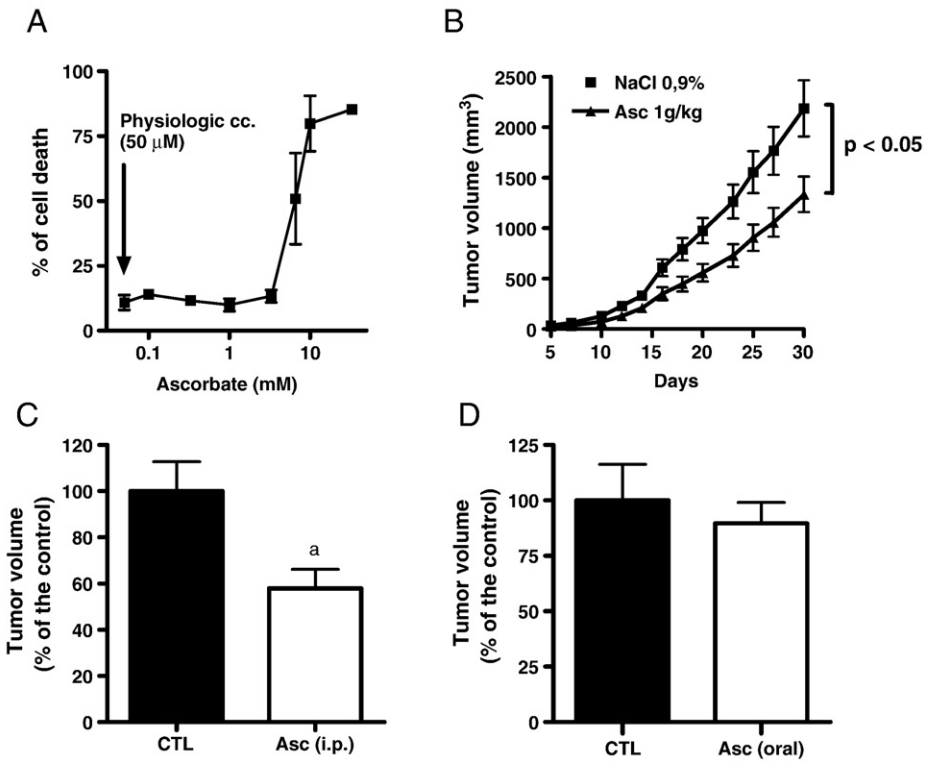

Fig. 5. Parenteral administration of ascorbate decreases tumor growth rate. (A) TLT cells (10^6/ml) were treated with various concentrations of ascorbate for 2 h, washed twice with PBS, and reincubated in fresh medium. Cell death was assessed 48 h after the exposure to ascorbate by measuring LDH leakage, as described under Materials and methods. Results are means of three separate experiments. (B) Animals ($n=20$ for each group) were implanted with TLT cells, as described under Materials and methods. Treatments were started 3 days after tumor implantation. Control mice received a daily ip injection of saline, whereas the ascorbate-treated group received a daily ip injection of ascorbate (1 g/kg). Tumor diameters were measured three times a week. Tumor growth in the ascorbate-treated group was significantly different from that of control ($p<0.05$), as assessed by two-way ANOVA. (C) Tumor volumes from the experiment presented in B were calculated at the end of the experiment (day 35). [a]$p<0.01$ versus control. (D) Animals ($n=11$ for each group) were implanted with TLT cells, as previously described. Treatments were started 1 week before tumor implantation and consisted of fresh water (control) or fresh water containing 6 g/L of ascorbate. Tumor volumes were calculated at the end of the experiment (day 24).

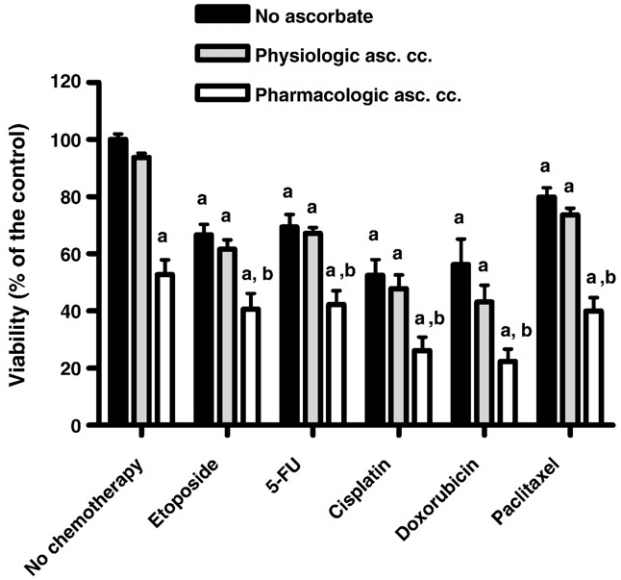

Fig. 6. Pharmacologic concentrations of ascorbate do not inhibit the activity of chemotherapies but rather reinforce their cytolytic effect. MCF7 cells (10,000) were treated with 50 μM or 4.5 mM ascorbate (= EC_{50}) for 2 h, either in the absence or in the presence of various chemotherapeutic agents used at the following concentrations: etoposide, 38.8 μM; 5-fluorouracil (5-FU), 4.8 μM; cisplatin, 51.5 μM; doxorubicin, 104.4 nM; paclitaxel, 2.8 nM. Cells were then washed twice with PBS and reincubated in fresh medium containing the chemotherapeutic agents at their respective EC_{50}. Viability was assessed by MTT assay 48 h after, as described under Materials and methods. Results are means of three separate experiments performed in quadruplicate. [a]$p<0.05$ (at least) versus no chemotherapy and [b]$p<0.05$ (at least) versus chemotherapy alone.

in Fig. 6, the combination of the EC_{50} of ascorbate and chemotherapy was more effective at killing MCF7 cells than either chemotherapy or ascorbate alone, whatever the chemotherapy used. Similar data were obtained with DU145 and T24 cells (data not shown). The physiologic concentration of ascorbate had no effect in every case.

Discussion

The use of ascorbate in cancer therapy has been proposed for more than 50 years but its efficacy is still a matter of controversy. Actually, as for many other unconventional anticancer agents, the early phase research was not or was inappropriately performed. As a consequence, the various parameters usually defined in these studies (doses, routes of administration, optimal schedule) were not correctly defined, leading to mixed results and controversy [35]. Fortunately, recent pharmacokinetic data have shed a new light on the pharmacokinetics of ascorbate because they show that, depending on the route of administration, the concentrations as well as the effects of ascorbate are dramatically different [11,12]. Thus, as confirmed by the results presented in this paper, the oral supplementation of ascorbate leads only to physiological blood concentrations, whereas parenteral administration allows pharmacologic concentrations of ascorbate, which are highly cytotoxic for various cancer cell lines. This observation may have a critical importance considering that previous clinical studies performed on ascorbic acid used different protocols and obtained different results [11,12]. Thus, the original studies of Pauling and Cameron used both iv and oral ascorbate [1,2], whereas the following double-blind placebo-controlled studies used only oral ascorbate [9,10]. The route of administration is probably not the only element that explains differences between the results of each study

(Pauling and Cameron studies were neither randomized nor placebo controlled) but, owing to the particular pharmacokinetics of ascorbic acid, it is clear that these studies are not comparable. Actually, our in vivo results confirm that the oral supplementation of ascorbate has no anticancer effect, as previously reported [9,10]. In contrast, the parenteral administration of ascorbate (1 g per kilogram of body weight, ip) was able to significantly decrease tumor growth in TLT-bearing mice, a result that has been recently observed in different models of aggressive tumor xenografts in mice [21]. The results we obtained suggest that ascorbate slows down but does not suppress tumor growth, an observation that could explain why, in the absence of a control group, no objective anticancer response was observed during the first phase I clinical trial of iv ascorbate in advanced cancer patients [25]. Nevertheless, it should be noted that ascorbate doses greater than 1 g/kg can be administered ip in animals (up to 4 g/kg) [21]. These doses would likely produce higher plasmatic concentrations of ascorbate and could have better effects on decreasing tumor growth.

Our in vitro results, as well as those obtained by others [18,36], support the idea that ascorbate induces the production of extracellular hydrogen peroxide, leading to oxidative stress and necrotic cell death, a death pathway that is interesting if we consider the multiple apoptotic defects usually exhibited by cancer cells [37]. The pro-oxidant activity of ascorbate is quite surprising given that this compound is generally considered an antioxidant. Actually, the precise mechanism by which ascorbate generates hydrogen peroxide in the extracellular medium is still unclear [38,39]. Indeed, ascorbate does not readily react with oxygen to produce reactive oxygen species, but it readily donates an electron to redox-active transition metal ions (such as iron and copper). These reduced metals can therefore react with oxygen to produce superoxide ions which, in turn, may dismutate to produce H_2O_2 [40]. However, extracellular chelators failed to protect against ascorbate toxicity, a result that was also described by others [36]. To explain this, Chen et al. have postulated the existence of extracellular metalloprotein catalysts present in the serum that could participate in hydrogen peroxide production by ascorbate [18], although precise identities of the proteins responsible remain unknown.

On the other hand, our results point out the role played by intracellular redox-active metal ions in ascorbate-mediated cell death. Indeed, preincubation with DFO, a cell-permeative metal chelator, had a protective effect against ascorbate toxicity, a result that was already obtained by Duarte et al. in genotoxicity assays [36]. The rationale would be that an intracellular reaction between hydrogen peroxide and redox-active metal ions would generate more reactive oxygen species, leading to an increased toxicity.

In vivo, the site of hydrogen peroxide production seems to be a critical element. Indeed, because red blood cells exhibit both catalase and glutathione peroxidase activities, ascorbate toxicity is completely inhibited in the presence of blood, which efficiently detoxifies hydrogen peroxide [18]. Therefore, the generation of hydrogen peroxide by ascorbate in vivo is possible only in extracellular fluids, as nicely demonstrated by Chen et al. [29]. Interestingly, no evident sign of toxicity has been recorded in vivo [25] and normal cells seem to be resistant to pharmacologic concentrations of ascorbate in vitro [18,21]. The origin of this difference in sensitivity between normal and cancer cells is unknown but various hypotheses have been formulated. Thus, oncogenic transformation has been reported to induce a higher basal status of intracellular ROS [41–43] associated with a greater sensitivity toward oxidative stress [44,45]. Alternatively, a low antioxidant status has been described in various cancer cell lines that could also participate in their sensitivity to ROS [46–49].

Up to now, the potential applications of parenteral administration of ascorbate in cancer treatment are speculative. A first conclusion is that ascorbate does not suppress but rather decreases tumor growth rate, as shown by preclinical and clinical data [21,25]. Owing to the absence of important side effects, an interesting perspective consists in its combination with other cytotoxic agents, as already suggested by several studies [19,20,50–52]. According to that strategy, our results show that ascorbate does not inhibit but rather enhances the activity of five important chemotherapeutic drugs (etoposide, cisplatin, 5-fluorouracil, doxorubicin, and paclitaxel). Because ascorbate generates an oxidative stress that preferentially targets cancer cells, it could be used as a modulator of the tumor redox status, a parameter known to be critical for the response to anticancer treatments [53,54]. This provided the rationale for its successful use in combination with arsenic trioxide (Trisenox), as observed both in preclinical [55–57] and in clinical studies [58,59]. Taken together, our results confirm previous work showing that the route of administration has a critical importance in the effects of ascorbate [12] and that pharmacologic concentrations of ascorbate possess anticancer properties [18,21]. They also highlight the putative interest of pharmacologic doses of ascorbate in cancer therapy although further evaluations are warranted to define the appropriate clinical applications.

Acknowledgments

The authors thank Isabelle Blave and Véronique Alleys for their excellent technical assistance. They are also deeply indebted to Roger Verbeeck for his valuable advice concerning the pharmacokinetic analysis, as well as his careful reading of the manuscript. This work was supported by a grant from the Belgian Fonds National de la Recherche Scientifique (FNRS-FRSM Grant 3.4594.04.F) and by the Fonds Spéciaux de Recherche, Université Catholique de Louvain. Julien Verrax is a FNRS postdoctoral researcher.

References

[1] Cameron, E.; Pauling, L. Supplemental ascorbate in the supportive treatment of cancer: prolongation of survival times in terminal human cancer. *Proc. Natl. Acad. Sci. USA* **73:**3685–3689; 1976.
[2] Cameron, E.; Pauling, L. Supplemental ascorbate in the supportive treatment of cancer: reevaluation of prolongation of survival times in terminal human cancer. *Proc. Natl. Acad. Sci. USA* **75:**4538–4542; 1978.
[3] Cameron, E.; Pauling, L.; Leibovitz, B. Ascorbic acid and cancer: a review. *Cancer Res* **39:**663–681; 1979.
[4] Bodansky, O.; Wroblewski, F.; Markardt, B. Concentrations of ascorbic acid in plasma and white blood cells of patients with cancer and noncancerous chronic disease. *Cancer* **5:**678–684; 1952.
[5] Gonçalves, T.; Erthal, F.; Corte, C.; Müller, L.; Piovezan, C.; Nogueira, C.; Rocha, J. Involvement of oxidative stress in the pre-malignant and malignant states of cervical cancer in women. *Clin. Biochem* **38:**1071–1075; 2005.
[6] Mayland, C.; Bennett, M.; Allan, K. Vitamin C deficiency in cancer patients. *Palliat. Med* **19:**17–20; 2005.
[7] Georgiannos, S.; Weston, P.; Goode, A. Micronutrients in gastrointestinal cancer. *Br. J. Cancer* **68:**1195–1198; 1993.
[8] Weijl, N.; Elsendoorn, T.; Lentjes, E.; Hopman, G.; Wipkink-Bakker, A.; Zwinderman, A.; Cleton, F.; Osanto, S. Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatin-based chemotherapy: a randomised, double-blind, placebo-controlled study. *Eur. J. Cancer* **40:**1713–1723; 2004.
[9] Creagan, E.; Moertel, C.; O'Fallon, J.; Schutt, A.; O'Connell, M.; Rubin, J.; Frytak, S. Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer: a controlled trial. *N. Engl. J. Med* **301:**687–690; 1979.
[10] Moertel, C.; Fleming, T.; Creagan, E.; Rubin, J.; O'Connell, M.; Ames, M. High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy: a randomized double-blind comparison. *N. Engl. J. Med* **312:**137–141; 1985.
[11] Padayatty, S.; Levine, M. Reevaluation of ascorbate in cancer treatment: emerging evidence, open minds and serendipity. *J. Am. Coll. Nutr* **19:**423–425; 2000.
[12] Padayatty, S.; Sun, H.; Wang, Y.; Riordan, H.; Hewitt, S.; Katz, A.; Wesley, R.; Levine, M. Vitamin C pharmacokinetics: implications for oral and intravenous use. *Ann. Intern. Med* **140:**533–537; 2004.
[13] Levine, M.; Conry-Cantilena, C.; Wang, Y.; Welch, R.; Washko, P.; Dhariwal, K.; Park, J.; Lazarev, A.; Graumlich, J.; King, J.; Cantilena, L. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc. Natl. Acad. Sci. USA* **93:**3704–3709; 1996.
[14] Levine, M.; Wang, Y.; Padayatty, S.; Morrow, J. A new recommended dietary allowance of vitamin C for healthy young women. *Proc. Natl. Acad. Sci. USA* **98:** 9842–9846; 2001.
[15] Prasad, K. Modulation of the effects of tumor therapeutic agents by vitamin C. *Life Sci* **27:**275–280; 1980.

[16] De Laurenzi, V.; Melino, G.; Savini, I.; Annicchiarico-Petruzzelli, M.; Finazzi-Agrò, A.; Avigliano, L. Cell death by oxidative stress and ascorbic acid regeneration in human neuroectodermal cell lines. *Eur. J. Cancer* **31A:**463–466; 1995.
[17] Kang, J.; Cho, D.; Kim, Y.; Hahm, E.; Yang, Y.; Kim, D.; Hur, D.; Park, H.; Bang, S.; Hwang, Y.; Lee, W. l-Ascorbic acid (vitamin C) induces the apoptosis of B16 murine melanoma cells via a caspase-8-independent pathway. *Cancer Immunol. Immunother* **52:**693–698; 2003.
[18] Chen, Q.; Espey, M.; Krishna, M.; Mitchell, J.; Corpe, C.; Buettner, G.; Shacter, E.; Levine, M. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc. Natl. Acad. Sci. USA* **102:**13604–13609; 2005.
[19] Verrax, J.; Stockis, J.; Tison, A.; Taper, H.; Calderon, P. Oxidative stress by ascorbate/menadione association kills K562 human chronic myelogenous leukaemia cells and inhibits its tumour growth in nude mice. *Biochem. Pharmacol* **72:**671–680; 2006.
[20] Verrax, J.; Vanbever, S.; Stockis, J.; Taper, H.; Calderon, P. Role of glycolysis inhibition and poly(ADP-ribose) polymerase activation in necrotic-like cell death caused by ascorbate/menadione-induced oxidative stress in K562 human chronic myelogenous leukemic cells. *Int. J. Cancer* **120:**1192–1197; 2007.
[21] Chen, Q.; Espey, M.; Sun, A.; Pooput, C.; Kirk, K.; Krishna, M.; Khosh, D.; Drisko, J.; Levine, M. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proc. Natl. Acad. Sci. USA* **105:**11105–11109; 2008.
[22] Wroblewski, F.; Ladue, J. Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med* **90:**210–213; 1955.
[23] Emadi-Konjin, P.; Verjee, Z.; Levin, A.; Adeli, K. Measurement of intracellular vitamin C levels in human lymphocytes by reverse phase high performance liquid chromatography (HPLC). *Clin. Biochem* **38:**450–456; 2005.
[24] Casciari, J.; Riordan, N.; Schmidt, T.; Meng, X.; Jackson, J.; Riordan, H. Cytotoxicity of ascorbate, lipoic acid, and other antioxidants in hollow fibre in vitro tumours. *Br. J. Cancer* **84:**1544–1550; 2001.
[25] Hoffer, L.; Levine, M.; Assouline, S.; Melnychuk, D.; Paddayatty, S.; Rosadiuk, K.; Rousseau, C.; Robitaille, L.; Miller, W. J. Phase I clinical trial of i.v. ascorbic acid in advanced malignancy. *Ann. Oncol* **19:**1969–1974; 2008.
[26] Murphy, J.; Ravi, N.; Byrne, P.; McDonald, G.; Reynolds, J. Neither antioxidants nor COX-2 inhibition protect against esophageal inflammation in an experimental model of severe reflux. *J. Surg. Res* **142:**20–27; 2007.
[27] Pincemail, J.; Siquet, J.; Chapelle, J.; Cheramy-Bien, J.; Paulissen, G.; Chantillon, A.; Christiaens, G.; Gielen, J.; Limet, R.; Defraigne, J. Determination of plasma concentrations of antioxidants, antibodies against oxidized LDL, and homocysteine in a population sample from Liège. *Ann. Biol. Clin. (Paris)* **58:**177–185; 2000.
[28] Myint, P.; Luben, R.; Welch, A.; Bingham, S.; Wareham, N.; Khaw, K. Plasma vitamin C concentrations predict risk of incident stroke over 10 y in 20 649 participants of the European Prospective Investigation into Cancer Norfolk prospective population study. *Am. J. Clin. Nutr* **87:**64–69; 2008.
[29] Chen, Q.; Espey, M.; Sun, A.; Lee, J.; Krishna, M.; Shacter, E.; Choyke, P.; Pooput, C.; Kirk, K.; Buettner, G.; Levine, M. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc. Natl. Acad. Sci. USA* **104:**8749–8754; 2007.
[30] Taper, H.; Woolley, G.; Teller, M.; Lardis, M. A new transplantable mouse liver tumor of spontaneous origin. *Cancer Res* **26:**143–148; 1966.
[31] Ansiaux, R.; Baudelet, C.; Cron, G.; Segers, J.; Dessy, C.; Martinive, P.; De Wever, J.; Verrax, J.; Wauthier, V.; Beghein, N.; Grégoire, V.; Buc Calderon, P.; Feron, O.; Gallez, B. Botulinum toxin potentiates cancer radiotherapy and chemotherapy. *Clin. Cancer Res* **12:**1276–1283; 2006.
[32] Crokart, N.; Jordan, B.; Baudelet, C.; Cron, G.; Hotton, J.; Radermacher, K.; Grégoire, V.; Beghein, N.; Martinive, P.; Bouzin, C.; Feron, O.; Gallez, B. Glucocorticoids modulate tumor radiation response through a decrease in tumor oxygen consumption. *Clin. Cancer Res* **13:**630–635; 2007.
[33] Frérart, F.; Sonveaux, P.; Rath, G.; Smoos, A.; Meqor, A.; Charlier, N.; Jordan, B.; Saliez, J.; Noël, A.; Dessy, C.; Gallez, B.; Feron, O. The acidic tumor microenvironment promotes the reconversion of nitrite into nitric oxide: towards a new and safe radiosensitizing strategy. *Clin. Cancer Res* **14:**2768–2774; 2008.
[34] Jordan, B.; Grégoire, V.; Demeure, R.; Sonveaux, P.; Feron, O.; O'Hara, J.; Vanhulle, V.; Delzenne, N.; Gallez, B. Insulin increases the sensitivity of tumors to irradiation: involvement of an increase in tumor oxygenation mediated by a nitric oxide-dependent decrease of the tumor cells oxygen consumption. *Cancer Res* **62:**3555–3561; 2002.
[35] Vickers, A.; Kuo, J.; Cassileth, B. Unconventional anticancer agents: a systematic review of clinical trials. *J. Clin. Oncol* **24:**136–140; 2006.
[36] Duarte, T.; Almeida, G.; Jones, G. Investigation of the role of extracellular H_2O_2 and transition metal ions in the genotoxic action of ascorbic acid in cell culture models. *Toxicol. Lett* **170:**57–65; 2007.
[37] Hanahan, D.; Weinberg, R. The hallmarks of cancer. *Cell* **100:**57–70; 2000.
[38] Carr, A.; Frei, B. Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J* **13:**1007–1024; 1999.
[39] Buettner, G.; Jurkiewicz, B. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat. Res* **145:**532–541; 1996.
[40] Frei, B.; Lawson, S. Vitamin C and cancer revisited. *Proc. Natl. Acad. Sci. USA* **105:**11037–11038; 2008.
[41] Vafa, O.; Wade, M.; Kern, S.; Beeche, M.; Pandita, T.; Hampton, G.; Wahl, G. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol. Cell* **9:**1031–1044; 2002.
[42] Sattler, M.; Verma, S.; Shrikhande, G.; Byrne, C.; Pride, Y.; Winkler, T.; Greenfield, E.; Salgia, R.; Griffin, J. The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells. *J. Biol. Chem* **275:**24273–24278; 2000.
[43] Laurent, A.; Nicco, C.; Chéreau, C.; Goulvestre, C.; Alexandre, J.; Alves, A.; Lévy, E.; Goldwasser, F.; Panis, Y.; Soubrane, O.; Weill, B.; Batteux, F. Controlling tumor growth by modulating endogenous production of reactive oxygen species. *Cancer Res* **65:**948–956; 2005.
[44] Trachootham, D.; Zhou, Y.; Zhang, H.; Demizu, Y.; Chen, Z.; Pelicano, H.; Chiao, P.; Achanta, G.; Arlinghaus, R.; Liu, J.; Huang, P. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* **10:**241–252; 2006.
[45] Trachootham, D.; Zhang, H.; Zhang, W.; Feng, L.; Du, M.; Zhou, Y.; Chen, Z.; Pelicano, H.; Plunkett, W.; Wierda, W.; Keating, M.; Huang, P. Effective elimination of fludarabine-resistant CLL cells by PEITC through a redox-mediated mechanism. *Blood* **112:**1912–1922; 2008.
[46] Oberley, T.; Oberley, L. Antioxidant enzyme levels in cancer. *Histol. Histopathol* **12:**525–535; 1997.
[47] Sun, Y.; Oberley, L.; Elwell, J.; Sierra-Rivera, E. Antioxidant enzyme activities in normal and transformed mouse liver cells. *Int. J. Cancer* **44:**1028–1033; 1989.
[48] Yang, J.; Lam, E.; Hammad, H.; Oberley, T.; Oberley, L. Antioxidant enzyme levels in oral squamous cell carcinoma and normal human oral epithelium. *J. Oral Pathol. Med* **31:**71–77; 2002.
[49] Verrax, J.; Cadrobbi, J.; Marques, C.; Taper, H.; Habraken, Y.; Piette, J.; Calderon, P. Ascorbate potentiates the cytotoxicity of menadione leading to an oxidative stress that kills cancer cells by a non-apoptotic caspase-3 independent form of cell death. *Apoptosis* **9:**223–233; 2004.
[50] Taper, H.; de Gerlache, J.; Lans, M.; Roberfroid, M. Non-toxic potentiation of cancer chemotherapy by combined C and K3 vitamin pre-treatment. *Int. J. Cancer* **40:**575–579; 1987.
[51] Kassouf, W.; Highshaw, R.; Nelkin, G.; Dinney, C.; Kamat, A. Vitamins C and K3 sensitize human urothelial tumors to gemcitabine. *J. Urol* **176:**1642–1647; 2006.
[52] Abdel-Latif, M.; Raouf, A.; Sabra, K.; Kelleher, D.; Reynolds, J. Vitamin C enhances chemosensitization of esophageal cancer cells in vitro. *J. Chemother* **17:**539–549; 2005.
[53] Kuppusamy, P.; Li, H.; Ilangovan, G.; Cardounel, A.; Zweier, J.; Yamada, K.; Krishna, M.; Mitchell, J. Noninvasive imaging of tumor redox status and its modification by tissue glutathione levels. *Cancer Res* **62:**307–312; 2002.
[54] Roshchupkina, G.; Bobko, A.; Bratasz, A.; Reznikov, V.; Kuppusamy, P.; Khramtsov, V. In vivo EPR measurement of glutathione in tumor-bearing mice using improved disulfide biradical probe. *Free Radic. Biol. Med.* **45:**312–320; 2008.
[55] Bahlis, N.; McCafferty-Grad, J.; Jordan-McMurry, I.; Neil, J.; Reis, I.; Kharfan-Dabaja, M.; Eckman, J.; Goodman, M.; Fernandez, H.; Boise, L.; Lee, K. Feasibility and correlates of arsenic trioxide combined with ascorbic acid-mediated depletion of intracellular glutathione for the treatment of relapsed/refractory multiple myeloma. *Clin. Cancer Res* **8:**3658–3668; 2002.
[56] Dai, J.; Weinberg, R.; Waxman, S.; Jing, Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. *Blood* **93:**268–277; 1999.
[57] Grad, J.; Bahlis, N.; Reis, I.; Oshiro, M.; Dalton, W.; Boise, L. Ascorbic acid enhances arsenic trioxide-induced cytotoxicity in multiple myeloma cells. *Blood* **98:**805–813; 2001.
[58] Berenson, J.; Boccia, R.; Siegel, D.; Bozdech, M.; Bessudo, A.; Stadtmauer, E.; Talisman Pomeroy, J.; Steis, R.; Flam, M.; Lutzky, J.; Jilani, S.; Volk, J.; Wong, S.; Moss, R.; Patel, R.; Ferretti, D.; Russell, K.; Louie, R.; Yeh, H.; Swift, R. Efficacy and safety of melphalan, arsenic trioxide and ascorbic acid combination therapy in patients with relapsed or refractory multiple myeloma: a prospective, multicentre, phase II, single-arm study. *Br. J. Haematol* **135:**174–183; 2006.
[59] Berenson, J.; Matous, J.; Swift, R.; Mapes, R.; Morrison, B.; Yeh, H. A phase I/II study of arsenic trioxide/bortezomib/ascorbic acid combination therapy for the treatment of relapsed or refractory multiple myeloma. *Clin. Cancer Res* **13:**1762–1768; 2007.