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

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Short title: Anticancer activity of pharmacologic doses of ascorbate

Abstract

Recently, it has been proposed that pharmacologic concentrations of ascorbate (vitamin C) can be reached by intravenous injection. Since 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 two-hour exposures, a protocol which mimics a parenteral use, we observed that pharmacologic concentrations of ascorbate killed very efficiently various cancer cell lines (EC50 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. On the contrary, 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 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 which exhibit interesting anticancer properties.

Key words: Ascorbate – Vitamin C – Oxidative stress – Cancer – Chemotherapy

Introduction

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 to 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 afterwards 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 these 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. On the contrary, 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 each study, 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. On the contrary,

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 appear strikingly more sensitive than normal cells [18]. Since we had previously used ascorbate to potentiate the cytotoxic effects of redox-active compounds [19, 20], we decided to study its own anticancer properties. The pharmacokinetics of ascorbate were investigated in mice, considering different routes of administration: oral, intravenous (i.v.) and intraperitoneal (i.p.). The cytolytic activity of ascorbate was then investigated *in vitro*, at various concentrations against different human cancer cell lines. Cells were incubated with ascorbate for only 2 hours in order to mimic a potential clinical i.v. use. Finally, the antitumoral activity of both oral and parenteral administration of ascorbate was investigated in a tumor-bearing mice 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), since N-acetyl-cysteine and catalase, two powerful antioxidants, were both able to completely suppress cell death. The cytolytic process involves intracellular reactive metals given that pre-incubation of cells with a cell-permeable chelator prevented cell death. *In vivo*, parenteral administration of ascorbate diminishes the growth of a murine hepatoma whereas oral administration of the same dosage (1g/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 x 10^5 cells per ml and the medium was changed at 48-72 h intervals. Human cancer cell lines (T24, DU145, MCF7, HepG2, Ishikawa) were cultured in high glucose DMEM (Dulbecco's modified eagle medium, Gibco) 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 % CO2 atmosphere at 100 % humidity. Phosphate Buffered Saline (PBS) was purchased from Gibco (Grand Island, NY).

Chemicals

Sodium ascorbate (vitamin C), N-acetylcysteine (NAC), carboxy-dichlorodihydrofluorescein diacetate (C-DCHF-DA), sanguinarine, ethylenediaminetetraacetic acid (EDTA), deferoxamine mesylate (DFO), diethylenetriaminepentaacetic acid (DTPA), protease inhibitor cocktail, catalase and dimethylsulfoxide (DMSO) were purchased from Sigma (St Louis, MO). The different 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). 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 wells) were plated in a 96-well plate and allowed to attach overnight. Forty-height hours after treatment, cells were washed two times with warm PBS and MTT containing medium was added to wells for 2 hours at 37°C. At the end of the incubation, 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 100 minus 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, allowing adherence overnight. They were then treated with ascorbate for 2 hours, washed with warm PBS and then fresh medium was added 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 reduced glutathione content was determined by using the GSH-glo Glutathione Assay (Madison, WI) according to the procedures described by the supplier.

Assessment of ROS formation

Carboxy-dichlorodihydrofluorescein diacetate (C-DCHF-DA) was used to detect ROS production. Cells were grown in Lab-Tek chambers plates and incubated either in the presence or in the absence of ascorbate for 30 minutes. They were then incubated at 37°C for 20 min in 1 ml of 1 μM of 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 (Mannheim, Germany) following manufacturer's instructions. Cells were then observed under a fluorescence microscope, as previously described.

Animals

6 Weeks–old female NMRI mice were used for all *in vivo* studies. Tumor implantation was done by injecting 10^6 of syngenic Transplantable Liver Tumor (TLT) hepatocarcinoma cells into the gastrocnemius muscle in the right hind limb of 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: (length x width2 x π)/6. Each procedure was approved by the local authorities according to national animal care regulations.

Ascorbate administration

Ascorbate-treated groups were injected either i.p. 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 i.p. and i.v. injections. For i.v. injections, animals were anaesthetized with sodium pentobarbital (Nembutal, 60 mg/kg weight) prior to a single injection in the tail vein. The ascorbate solutions were hypertonic, as 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 in 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 the one used for parenteral injections.

Blood samplings

Blood samples were obtained from the lateral saphenous vein and collected in eppendorf vials containing tripotassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant. They were kept on ice before being centrifuged at 2000 g at room temperature for 5 minutes to obtain plasma. Plasma samples were then stored at –80°C and analyzed within one week.

Ascorbate quantification

Plasma samples were deproteinized by adding half a volume of a solution containing 20 % metaphosphoric acid and 6 mM of EDTA. They were then centrifuged at 13 000 g at 4°C for 10 minutes. 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 (Milford, USA). Samples were transferred to the column by a Rheodyne 7125 injector

(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 pre-column (Alltech, Breda, The Netherlands). The mobile phase contained 2 mM of EDTA and consisted of KH_2PO_4 0,2 M 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 has been used for all calculations (GraphPad Software Inc., San Diego, California, USA). EC50 values were determined by nonlinear regression. Pharmacokinetic data were analyzed by using a non-compartmental analysis and the following parameters were determined (when required): the initial peak concentration (C °p), peak concentration in plasma (Cmax), time to Cmax (Tmax), areas under curve (AUC $0\rightarrow\infty$), clearance (CL), fraction of drug absorbed (F) and elimination half-life (t1/2). Both the initial peak concentration (C°p) and the elimination rate constant (k) were estimated by using linear regression performed on the logarithm of plasma concentrations. The half-life (t1/2) was calculated by the equation $t1/2 = \ln 2/k$. The AUC values (up to 120 minutes) were calculated using GraphPad Prism, and the extrapolation to infinity was calculated by dividing the last measured concentration by k. The clearance (CL) was calculated by dividing the dose by the AUC (multiplied by the bioavailability in the case of i.p. administration). The fraction of ascorbate absorbed after i.p. injection (F)

was calculated by dividing the AUC after i.p. administration by the AUC after the i.v. 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 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 i.v. or i.p. injections.

First of all, the pharmacokinetics of ascorbate have been investigated. Pharmacokinetic profiles were obtained from 6 mice receiving either i.v. (as a bolus) or i.p. 1 g/kg of sodium ascorbate, a dose which 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 minutes after the injection (Figure 1A). From the non-compartmental analysis, the initial peak concentration (C°p) was estimated to be 22 ± 3 mM (Table 1). Intraperitoneal injections resulted in a peak plasma concentration of 7 ± 1 mM, 30 minutes after injection (Figure 1B). Since the average basal ascorbate concentration in these animals was only 12 ± 3 μM, this means that a 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, with 40 ± 8 and 54 ± 9 minutes, for i.v. and i.p. respectively. Ascorbate levels returned to normal values (19 ± 2 μM, n=4) 24 hours after an i.p. injection, suggesting that no accumulation occurs. From the respective area under curve (AUCs) values, the bioavailability (F) for i.p. administration was estimated to 0.62 (Table 1).

The effect of oral supplementation was also investigated in a group of 6 animals treated with 1 g/kg of ascorbate in drinking water for 4 weeks. At the end of the treatment, a significant three-fold increase of ascorbate concentration was found (Figure 1C), consistent with previously published results in rats [26]. However, plasma ascorbate

concentrations following oral administrations remained below 50 µM, which is more than hundred 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.

Since 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 (Figure 2A). A two-hour exposure was used for all the *in vitro* experiments, in order to mimic a clinical i.v. use and cellular viability was checked 48 hours later by the MTT reduction assay. A similar profile of toxicity was observed in all cell lines tested and the different EC50 were ranging from 3 to 7 mM. Interestingly, these values were neither related to 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 i.v.), which allows, for at least 1 hour, pharmacologic concentrations above any EC50. The toxicity of pharmacologic concentrations of ascorbate was further confirmed in a clonal survival assay in which we observed that a two-hour exposure to 5 mM ascorbate led to a decrease of 61 to 99 % in the number of colonies (Figure 2B).

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

Since 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 reactive oxygen species (ROS) in the cytotoxic process. We observed that N-acetylcysteine (NAC) and catalase (CAT), two H_2O_2 scavengers, completely suppressed cell death in all cell lines tested (Figure 3A). It should be underlined that catalase had also a protective effect in the clonal survival assay, whereas heat-inactivated catalase was ineffective (data not shown). Since catalase is likely membrane impermeable, 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 carboxy-dichlorodihydrofluorescein diacetate (C-DCHF-DA). Our results show that an increase of intracellular ROS can be detected as soon as 30 minutes after the exposure of cells to ascorbate (Figure 3B). Supporting the occurrence of an oxidative stress, we observed decreases of respectively 20 and 40 % in GSH and ATP four hours after the exposure to ascorbate, in all the cell lines tested (T24, DU145, MCF7 and Ishikawa) (data not shown). The addition of different chelators suggests that intracellular metals participate in ascorbate toxicity. Indeed, pre-incubation with deferoxamine mesylate (DFO), a cell-permeable metal chelator, prevented the loss of viability of tumor cells exposed to pharmacologic concentration of ascorbate (Figure 3C). On the contrary, two cell-impermeable chelators, namely ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) failed to protect against ascorbate toxicity

(Figure 3D). Confirming the importance of intracellular metals in ascorbate toxicity, we observed that DFO had also a protective effect in the clonal survival assay (Figure 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 (Figure 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 by using a double annexin-V and propidium iodide staining. As shown in Figure 4B, ascorbate-treated cells exhibit a clear necrotic profile (annexin-V and propidium iodide positive), as soon as eight hours 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].

Parenteral administration of ascorbate decreases tumor growth rate.

Since pharmacologic ascorbate concentrations can be reached *in vivo,* and as these latter induce cell death in various cancer cell lines *in vitro*, we then looked for a putative anticancer effect in a tumor-bearing mice model. For that purpose, we chose the murine Transplantable Liver Tumor (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 towards ascorbate. As observed for other cell lines, TLT cells were efficiently killed by pharmacologic concentrations of ascorbate and presented an EC50 value of approximately 6 mM (Figure 5A). Our results show that a daily administration of ascorbate (1g/kg i.p.) significantly decreases tumor growth rate (Figure 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 % ($p<0.01$) (Figure 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 figure 5D, the oral supplementation of ascorbate in drinking water failed to induce any decrease of 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.

Since 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 EC50 values, either in the absence or in the presence of ascorbate for which two concentrations were assessed: 50 µM and the EC50 value. As shown in Figure 6, the combination between EC50 of ascorbate and chemotherapy was more effective to kill MCF7 cells than either chemotherapy or ascorbate alone, whatever the chemotherapy used. Similar data have been obtained with DU145 and T24 cells (data not shown). The physiological concentration of ascorbate had no effect in every case.

Discussion

The use of ascorbate in cancer therapy has been proposed for more than fifty 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 inappropriately performed. As a consequence, the different 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 since 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 i.v. 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, due 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]. On the contrary, the parenteral administration of ascorbate (1g per kilogram of body weight,

i.p.) 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 which could explain that, in the absence of a control group, no objective anticancer response was observed during the first phase I clinical trial of i.v. ascorbate in advanced cancer patients [25]. Nevertheless, it should be noted that ascorbate doses greater than 1g/kg can be administered i.p. in animals (up to 4g/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 as 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, on the contrary, 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]. Explaining 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, pre-incubations with DFO, a cell-permeable 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 appears as a critical element. Indeed, since 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 only possible 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 of sensitivity between normal and cancer cells is unknown but different hypotheses have been formulated. Thus, oncogenic transformation has been reported to induce a higher basal status of intracellular ROS [41-43] associated to a greater sensitivity towards 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 still speculative. A first conclusion is that ascorbate does not suppress but

rather decrease tumor growth rate, as shown by preclinical and clinical data [21, 25]. Due 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). Since 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 pre-clinical [55-57] and clinical studies [58, 59]. Taken together, our results confirm previous works 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 in order to define the appropriate clinical applications.

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List of abbreviations

ROS, reactive oxygen species; NAC, N-acetyl-cysteine; CAT, catalase; TLT, transplantable liver tumor; DMEM, Dulbecco's modified eagle medium; PBS, phosphate buffered saline; C-DCHF-DA, carboxy-dichlorodihydrofluorescein diacetate; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; i.p., intraperitoneal; i.v., intravenous; EDTA, ethylenediaminetetraacetic acid; DFO, deferoxamine mesylate; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; UV, ultraviolet; ANOVA, analysis of variance; AUC, area under curve; H_2O_2, hydrogen peroxide; C°p, initial peak concentration; Tmax, time of occurrence for maximum (peak) concentration; Cmax, maximum concentration; t½, half-life; CL, clearance; F, fraction of drug absorbed (bioavailability).

Legends

Figure 1. Pharmacologic concentrations of ascorbate are achieved by either i.v. or i.p. injections. *A*, Pharmacokinetic profile obtained after the i.v. administration (tail vein) of 1g/kg of ascorbate in mice. Data were obtained from 6 animals. Blood samples were taken at different times (5, 15, 30, 90 and 120 minutes), plasma was isolated and stored at –80°C, as described in the Materials and Methods section. The ascorbate levels were quantified within the week by using HPLC coupled with UV detection. Pharmacokinetic data were analyzed by a non-compartmental analysis. *B*, Pharmacokinetic profile obtained after the i.p. administration of 1g/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 in drinking water during 4 weeks at 6g/l, as described in the Material and Methods section. "a" means $p<0.001$ versus control.

Figure 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 hours, then washed twice with PBS and re-incubated in fresh medium. Viability was assessed by MTT 48 hours after the exposure to ascorbate, as described in the Material and Methods section. Results are mean of three separate experiments performed each in triplicate. *B*, Cells (500) were treated with 5 mM ascorbate for 2 hours, then washed twice with PBS and re-incubated in fresh medium for ten days. Colonies were then visualized by using crystal violet. Results are mean of two separate experiments (n=5). "a" means $p<0.001$ versus control.

Figure 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 hours either in the presence or in the absence of inhibitors. They were then washed twice with PBS and re-incubated in fresh medium. Viability was assessed by MTT 48 hours after the exposure to ascorbate, as described in the Material and Methods section. Results are mean of three separate experiments performed each in triplicate. "a" and "b" mean respectively $p<0.001$ and $p<0.01$ versus control. *B*, T24 cells (10 000) were seeded in Lab-Tek chambers as described in the Material and Methods section. They were then treated with 10 mM ascorbate for 30 minutes, then washed and incubated for a further 20 min in the presence of 1 µM of carboxy-DCFDA. *C and D*, DU145 cells (10 000) were first pre-incubated for three hours with different chelators used at the following concentrations: ethylenediaminetetraacetic acid (EDTA), 1mM; deferoxamine mesylate (DFO), 500 µM; diethylenetriaminepentaacetic acid (DTPA), 1mM. They were then washed twice with PBS and re-incubated in fresh medium containing 10 mM ascorbate for 2 hours (either in the presence or in the absence of chelators for the results shown in Figure D). At the end of the incubation, cells were washed twice with PBS and re-incubated in fresh medium. Viability was assessed by MTT, 48 hours after the exposure to ascorbate, as described in the Material and Methods section. Results are mean of three separate experiments performed each in quadruplicate. "a" means $p<0.001$ versus control. *E*, DU145 cells (500) were first pre-incubated for

three hours in the absence or in the presence of deferoxamine mesylate (DFO, 500 μM). They were then washed twice with PBS and then re-incubated in fresh medium containing 10 mM ascorbate for 2 hours. At the end of the incubation, cells were washed twice with PBS and re-incubated in fresh medium for ten days. Colonies were visualized by using crystal violet. Results are mean of two separate experiments performed in triplicate. “a” and “b” mean respectively $p<0.001$ and $p<0.01$ versus control.

Figure 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 hours, either in the presence or in the absence of 50 μM of a broad caspase inhibitor (Z-VAD-fmk), which was pre-incubated for 1 hour. They were then washed twice with PBS and re-incubated in fresh medium in the presence or in the absence of the caspase inhibitor. Viability was assessed by MTT 48 hours after the exposure to ascorbate, as described in the Material and Methods section. Results are mean of three separate experiments performed each in triplicate. “a” means $p<0.001$ versus control. *B*, T24 cells (10 000) were seeded in Lab-Tek chambers, allowed to attach and treated with 10 mM ascorbate for 2 hours, then washed and incubated for a further 6 hours. Sanguinarine (10 μM) was used as a positive control of apoptosis, as previously described [19]. Double staining with Annexin-V / Propidium iodide was performed as described in the Material and Methods section.

Figure 5. Parenteral administration of ascorbate decreases tumor growth rate. *A*, TLT cells (10^6/ml) were treated with various concentrations of ascorbate for 2 hours, then washed twice with PBS and re-incubated in fresh medium. Cell death was assessed 48

hours after the exposure to ascorbate by measuring LDH leakage, as described in the Material and Methods section. Results are mean of three separate experiments. *B*, Animals (n=20 for each group) were implanted with TLT cells, as described in the Material and Methods section. Treatments were started three days after tumor implantation. Control mice received a daily i.p. injection of saline, whereas ascorbate-treated group received a daily i.p. injection of ascorbate (1g/kg). Tumor diameters were measured three times a week. Tumor growth in 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 figure 5B were calculated at the end of the experiment (day 35). "a" means $p<0.01$ 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 6g/l of ascorbate. Tumor volumes were calculated at the end of the experiment (day 24).

Figure 6. Pharmacologic concentrations of ascorbate do not inhibit the activity of chemotherapies but rather reinforce their cytolytic effect. *A*, MCF7 cells (10 000) were treated with 50 µM or 4.5 mM of ascorbate (=EC50) for 2 hours, either in the presence or in the absence 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 re-incubated in fresh medium containing chemotherapeutic agents at their respective EC50. Viability was assessed by MTT 48 hours after, as described in the Material and Methods section. Results are mean of three separate experiments performed each in quadruplicate. "a"

means $p<0.05$ (at least) versus no chemotherapy and "b" means $p<0.05$ (at least) versus chemotherapy alone.

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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.

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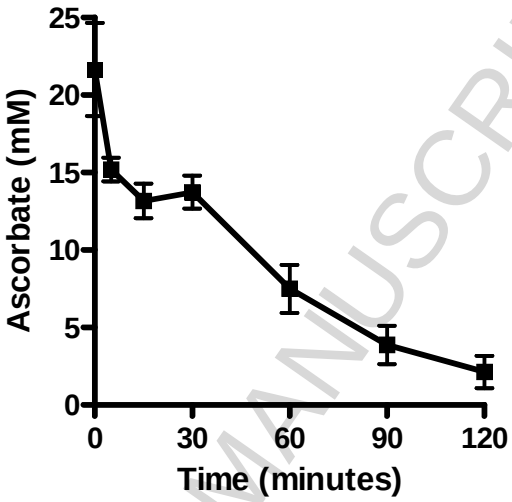
Figure 1. Pharmacologic concentrations of ascorbate are achieved by either i.v. or i.p. injections.

A.



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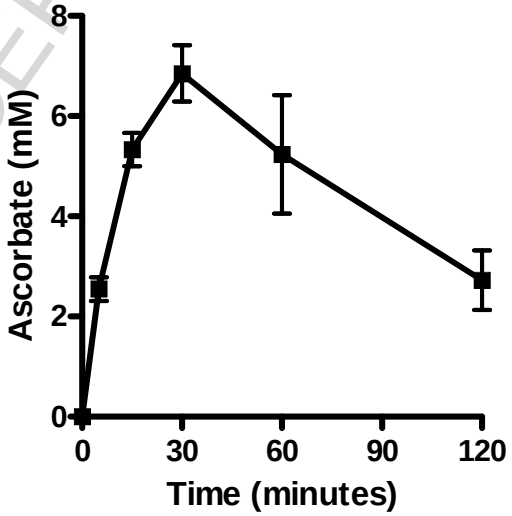

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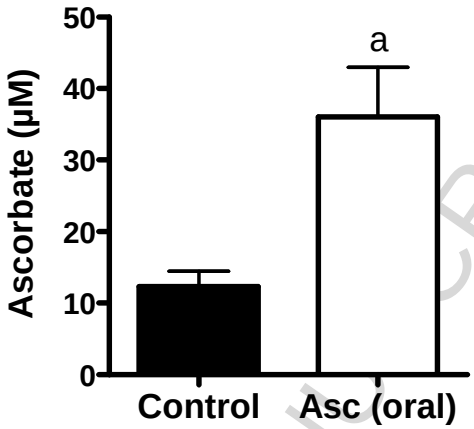
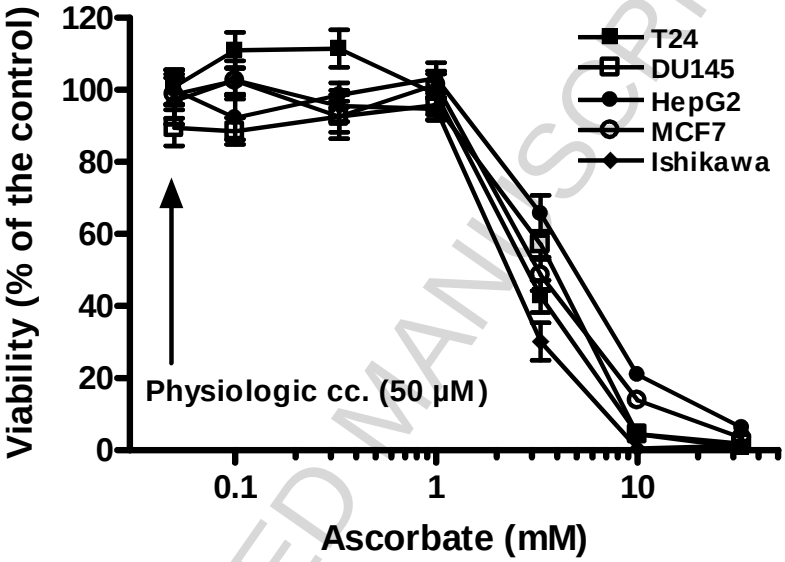


Figure 2. Various cancer cell lines are killed by pharmacologic concentrations of ascorbate.

A.



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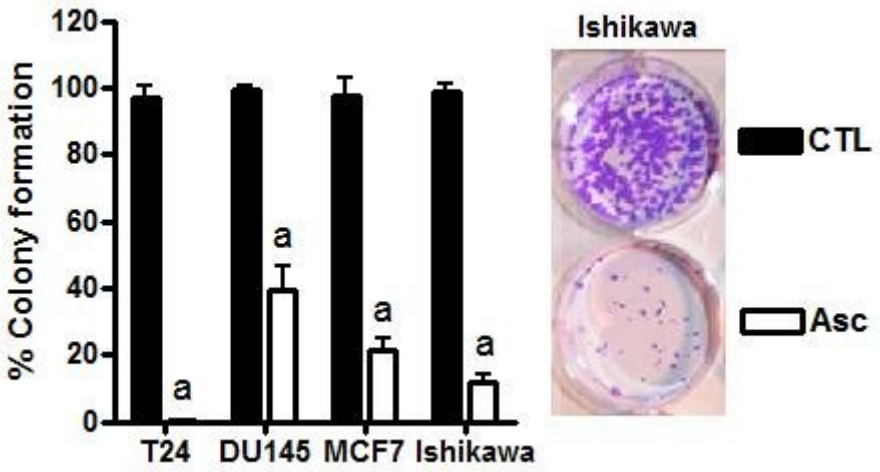
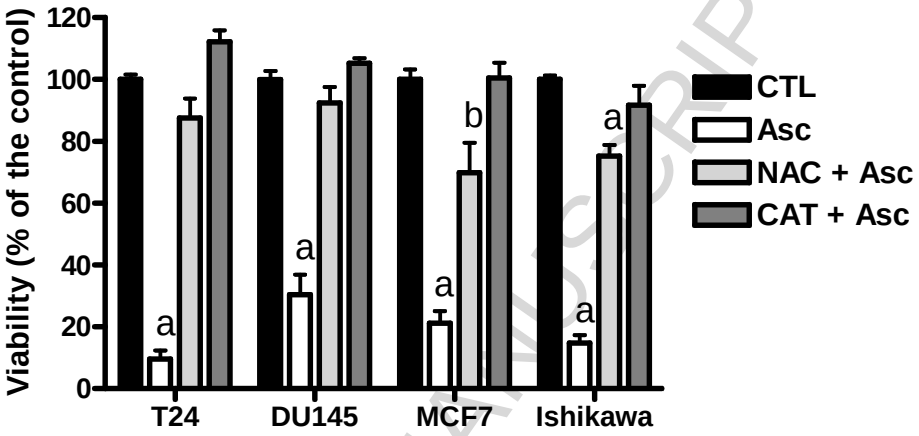


Figure 3. Pharmacologic concentrations of ascorbate generate extracellular hydrogen peroxide which reacts with intracellular metals.

A



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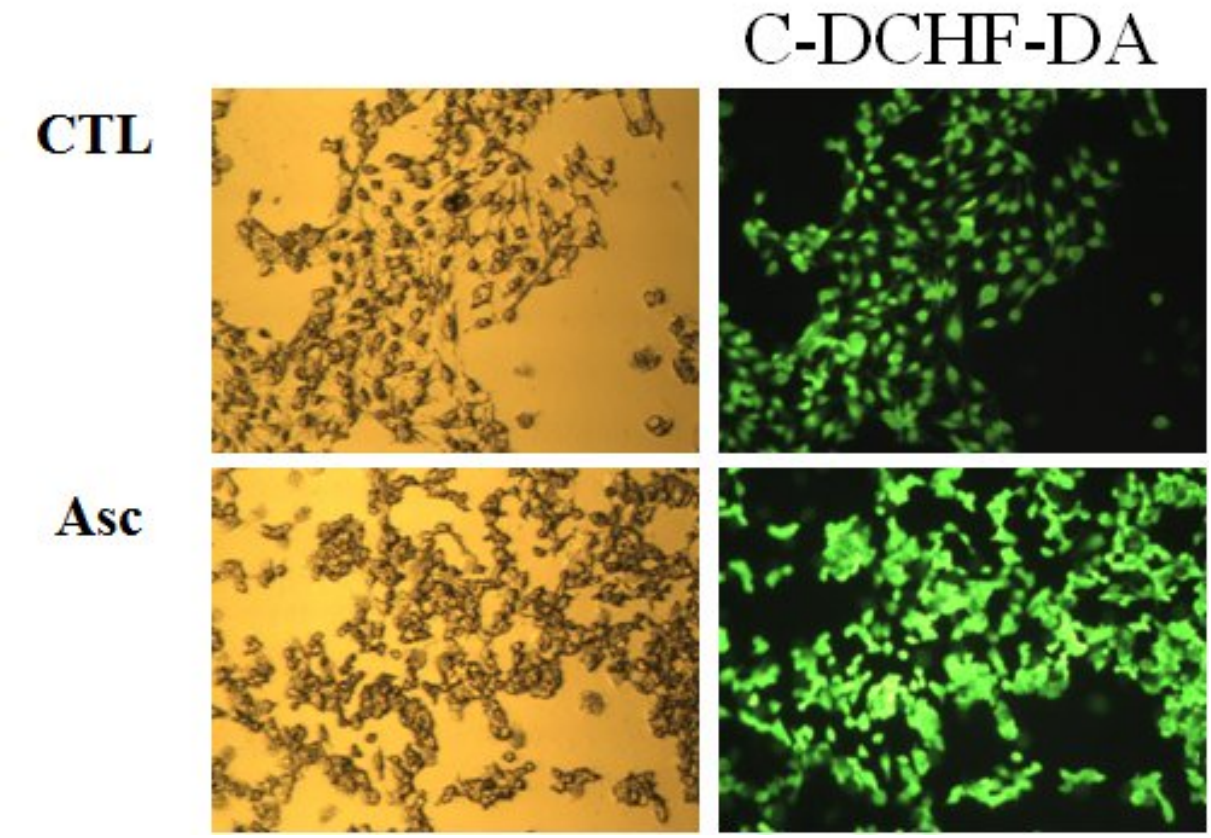

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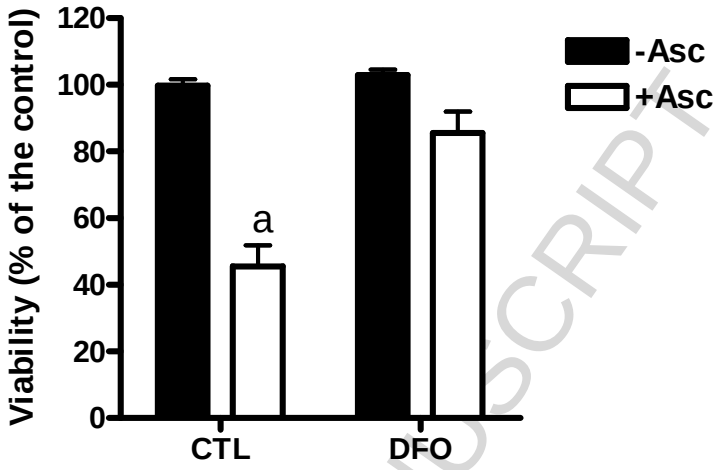

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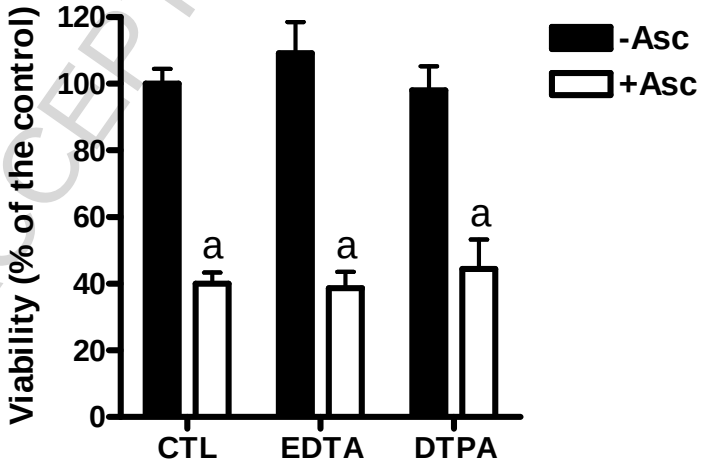

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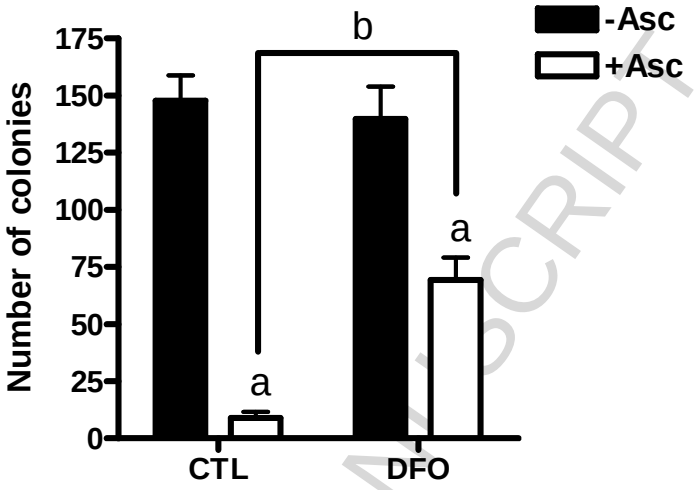
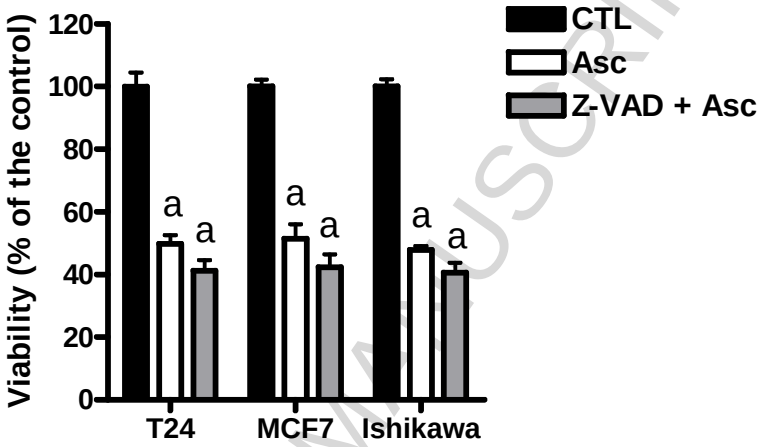


Figure 4. Cancer cells exposed to pharmacological concentrations of ascorbate die through a necrotic cell death.

A.



B.

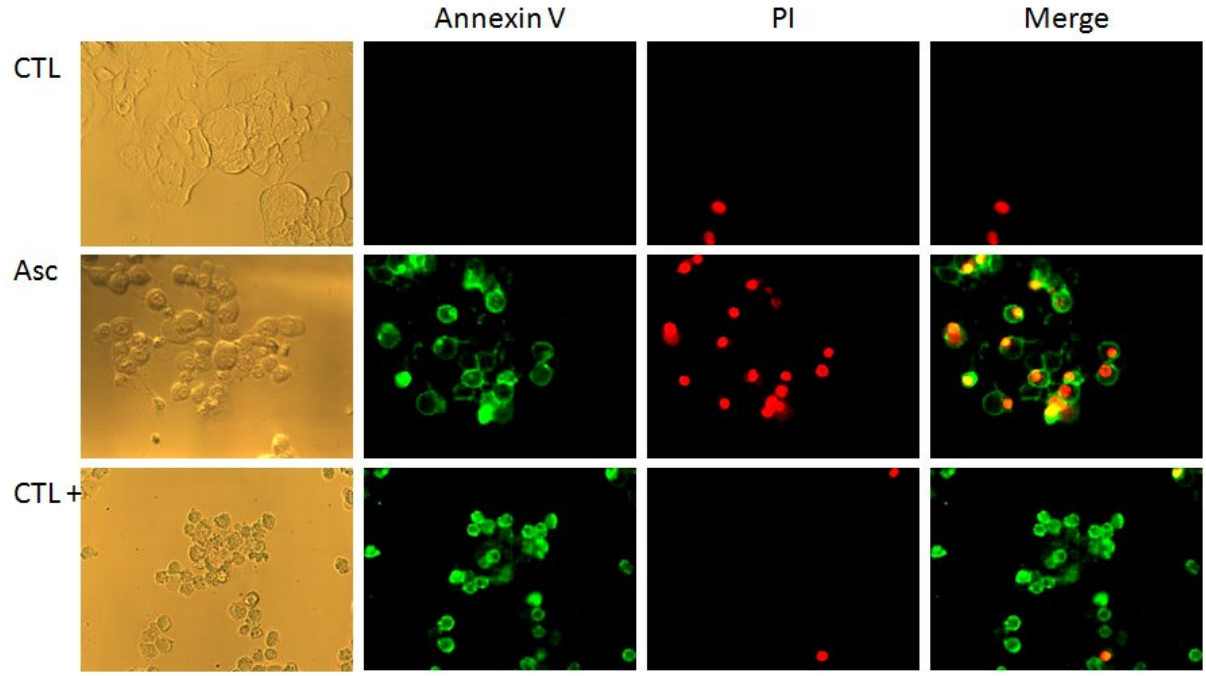
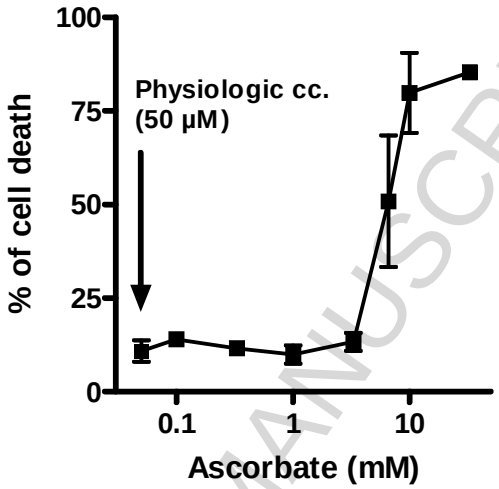
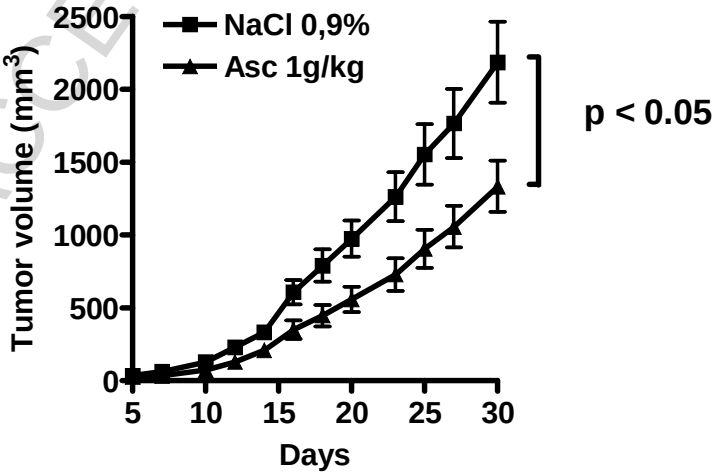


Figure 5 Parenteral administration of ascorbate decreases tumor growth rate.

A.



B.



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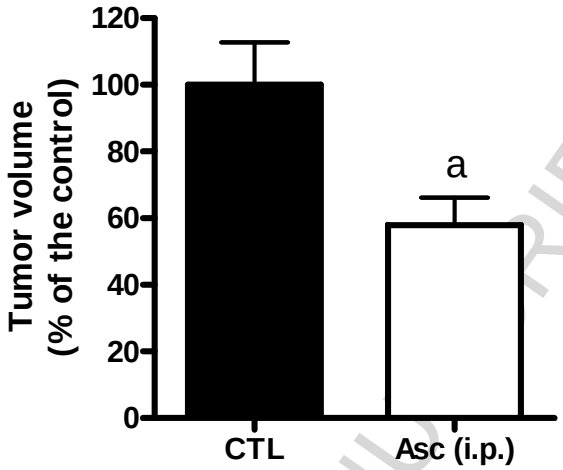

D

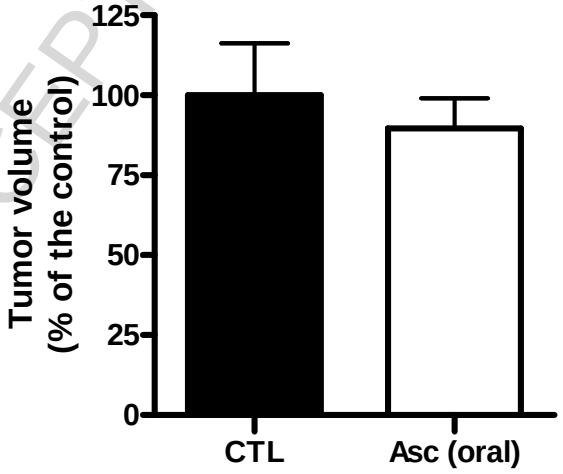

Figure 6. Pharmacologic concentrations of ascorbate do not inhibit the activity of chemotherapies but rather reinforce their cytolytic effect.

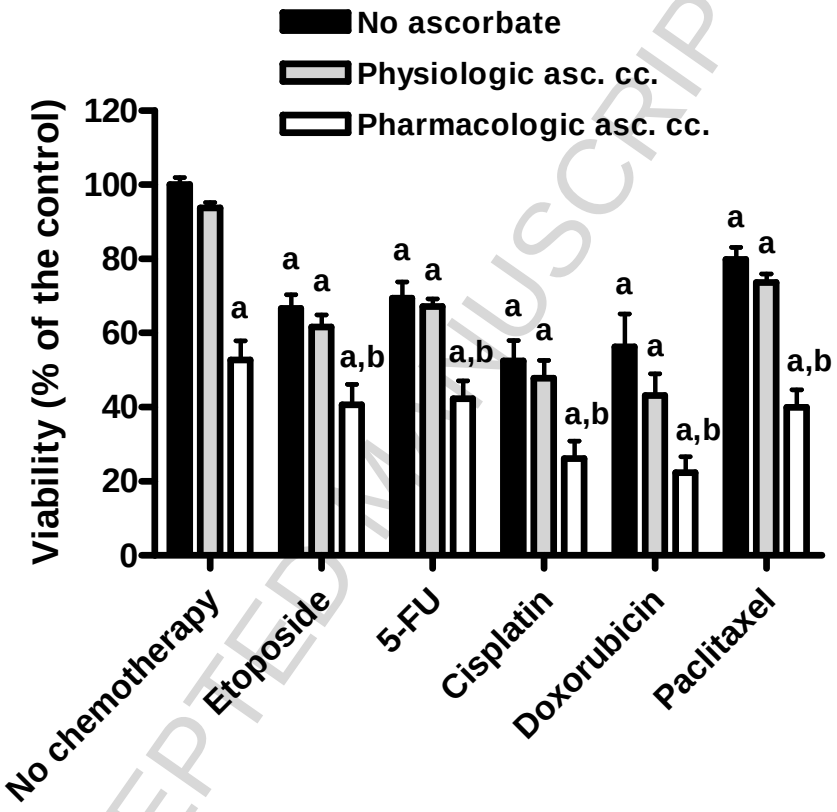

Table 1. Pharmacokinetic data of parenteral administration of ascorbate

Paremeter	**I.P.**	**I.V.**
C°p	/	22 +- 3 mM
Tmax	0.5 h	/
Cmax	7 +- 1 mM	/
t½	54 +- 9 min	40 +- 8 min
CL	9 +- 2 ml/h	8 +- 1 ml/h
AUC 0→∞	12 +- 3 mM*h	20 +- 2 mM*h
F	0.62	/

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

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