Ascorbate/menadione-induced oxidative stress kills cancer cells that express normal or mutated forms of the oncogenic protein Bcr-Abl. An in vitro and in vivo mechanistic study

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Summary Numerous studies suggest that generation of oxidative stress could be useful in cancer treatment. In this study, we evaluated, in vitro and in vivo, the antitumor potential of oxidative stress induced by ascorbate/menadione (asc/men). This combination of a reducing agent (ascorbate) and a redox active quinone (menadione) generates redox cycling leading to formation of reactive oxygen species (ROS). Asc/men was tested in several cell types including K562 cells (a stable human-derived leukemia cell line), freshly isolated leukocytes from patients with chronic myeloid leukemia, BaF3 cells (a murine pro-B cell line) transfected with Bcr-Abl and peripheral blood leukocytes derived from healthy donors. Although these latter cells were resistant to asc/men, survival of all the other cell lines was markedly reduced, including the BaF3 cells expressing either wild-type or mutated Bcr-Abl. In a standard in vivo model of subcutaneous tumor transplantation, asc/men provoked a significant delay in the proliferation of K562 and BaF3 cells expressing the T315I mutated form of Bcr-Abl. No effect of asc/men was observed when these latter cells were injected into blood of mice most probably because of the high antioxidant potential of red blood cells, as shown by in vitro experiments. We postulate that cancer cells are more sensitive to asc/men than healthy cells because of their lack of antioxidant enzymes, mainly catalase. The mechanism underlying this cytotoxicity involves the oxidative cleavage of Hsp90 with a subsequent loss of its chaperone function thus leading to degradation of wild-type and mutated Bcr-Abl protein.

Keywords Ascorbate · Bcr-Abl · Menadione · Oxidative stress · Hsp90 · Redox cycling

Introduction

Maintenance of a genetic instability within cancer cells requires the presence of reactive oxygen species (ROS) to induce DNA lesions (e.g. mutations), generating an ambiguous relationship between the quantity of ROS
required and the capacity of tumor cells to progress and to proliferate [1, 2]. On the other hand, since most cancer cells have low antioxidant capacities [3, 4], an extremely fragile redox equilibrium is created that can be easily overwhelmed by inducing oxidative stress. Hence, a successful therapeutic strategy could be the induction of cancer cell death using different unrelated oxidant compounds, like PEITC [5], Trisenox [6], Adaphostin [7] or ascorbate/menadione [4, 8–11]. We have previously reported that ascorbate/menadione (asc/men) is an ROS-generating system that induces cell death in a wide variety of cancer cell lines, most probably through a complex mechanism involving glycolysis inhibition [11], calcium homeostasis deregulation [12] and impairment of the chaperoning function of a key protein like Hsp90 [13]. The mechanism underlying ROS formation relies on ascorbate-driven menadione-redox cycling which generates H$_2$O$_2$ (Fig. 1), this compound is more toxic for cancer than for non-cancer cells because cancer cells lack antioxidant enzymes, such as catalase [14], and have high basal level of ROS.

We, therefore, postulated that induction of oxidative stress would impair critical functions in cancer cells. Indeed, in K562 cells, a stable human chronic myeloid leukemia (CML) cell line, an oxidant insult by asc/men provoked the cleavage of Hsp90 and subsequent impairment of its chaperone function, leading to the degradation of several client proteins, such as Bcr-Abl, Akt and RIP [13]. The oncogenic protein Bcr-Abl, a constitutively active protein tyrosine kinase, is responsible for the transformation of normal cells to CML cells [15–19]. Since the survival of these cells is mainly dependent on the activity of the Bcr-Abl oncogene, its inhibition by imatinib revolutionized the treatment of CML [20]. Nevertheless, because the Bcr-Abl gene is unstable [21], mutations in the kinase domain of Bcr-Abl do appear, the worst being T315I, the substitution of the threonine residue at position 315 by an isoleucine residue that renders Bcr-Abl-T315I bearing cells highly resistant to imatinib [22]. Since the stability of the Bcr-Abl protein relies on the chaperone activity of Hsp90 [23], we postulated that asc/men, by inducing Hsp90 cleavage and degradation of its client proteins, would kill cells expressing either wild-type or mutated forms of Bcr-Abl. The results reported in this study show that, under in vitro conditions and in solid tumor models, asc/men is active against all Bcr-Abl bearing cell lines whether or not they express the E255K or T315I mutations. Asc/men is less cytotoxic against peripheral blood leukocytes derived from healthy donors. We postulate that, because of their lack of antioxidant enzymes, mainly catalase, cancer cells are more sensitive to asc/men than healthy cells. The mechanism underlying the cytotoxicity of the asc/men combination involves the oxidative cleavage of Hsp90 with a subsequent loss of its chaperone function thus leading to the degradation of wild-type and mutated Bcr-Abl proteins.

**Material and methods**

**Chemicals and antibodies**

Menadione sodium bisulfite, sodium ascorbate, erythrosine B, ammonium chloride, dimethylsulfoxide and heparin sodium salt were purchased from Sigma (St Louis, MO). H$_2$O$_2$ (hydrogen peroxide) was purchased from Merck (Darmstadt, Germany). Imatinib mesylate (Gleevec®) was from Novartis (Basel, Switzerland). Polyclonal rabbit primary antibody against c-abl was purchased from Cell Signaling Technology (Danvers, MA), mouse monoclonal primary antibody against Hsp90 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal rabbit primary antibody against catalase was purchased from Chemicon (Billerica, MA), mouse monoclonal primary antibody against β-actin was purchased from Abcam (Cambridge, United Kingdom), mouse monoclonal primary antibody against phospho-tyrosines was purchased from

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**Fig. 1** The ascorbate-driven menadione redox cycling
Upstate (Billerica, MA). Rabbit secondary antibody was purchased from Chemicon (Billerica, MA), mouse secondary antibody was purchased from Dako (Glostrup, Denmark). Protease inhibitor cocktail was purchased from Sigma (St Louis, MO). DEPMPO was purchased from Radical Vision (Marseille, France). All other chemicals were ACS reagent grade.

Cell culture conditions

BaF3/Bcr-Ablwild-type (BaF3/Bcr-Abl-WT), BaF3/Bcr-AblE255K (BaF3/Bcr-Abl-E255K), and BaF3/Bcr-AblT315I (BaF3/Bcr-Abl-T315I) cell lines were a gift from Dr. K. Bhalla (MCG Cancer Center, Medical College of Georgia, Augusta, GA); they were maintained in RPMI1640 medium supplemented with 10% fetal calf serum, streptomycin 100 μg/ml, penicillin 100 IU/ml, and 1% non-essential amino acids solution (Gibco, Paisley, United Kingdom) at 37°C in humidified 5% CO2. The CML cell line K562 was purchased from the European cell culture collection (ECACC) and maintained in RPMI1640 medium supplemented with 10% fetal calf serum, streptomycin 100 μg/ml, penicillin 100 IU/ml, and gentamicin (50 μg/ml) at 37°C in humidified 5% CO2. Both K562 and BaF3 cells were incubated at a concentration of 1 × 10⁶ cells per ml. Inhibitors and/or asc/men (2 mM/10 μM) were added directly into the incubation media at the indicated times. Imatinib mesylate (imatinib) was used at a concentration of 10 μM.

Cell survival and proliferation assays

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH) both in the culture medium and in the cell pellet obtained after centrifugation according to the procedure of Wroblesky and Ladue, as previously described [11]. The results are expressed as the ratio of released activity to total activity.

Proliferation was measured by incubating the cells with or without asc/men for 48 h and then counting the number of viable cells (erythrosine B exclusion of non-viable cells) per unit of volume, allowing the rate of proliferation to be estimated. Results were expressed as a percentage of what was observed in control untreated cells.

Isolation of leukocytes

Blood samples were collected from either healthy volunteers or from patients diagnosed with CML, and then centrifuged at 750 g for 7 min. The upper phase (plasma) was eliminated and the phase containing the leukocytes was collected and washed three times with a buffer (NH₄Cl 155 mM, KHCO₃ 10 mM, EDTA 1 mM) that provokes the lysis of red blood cells (RBC). Cells were then counted and incubated in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin. Informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Bioethetical Human Assurance Committee (reference number 2009/10MAR/100, B40320096107).

Isolation of red blood cells

Mice were anesthetized and blood was taken by intracardiac puncture. Blood was anticoagulated with heparin and centrifuged at 500 g for 30 min. Red blood cells (RBC) were washed with PBS and resuspended in appropriate culture medium. They were used at a final concentration of 1% hematocrit.

Western blot analyses

Appropriate protein amounts (40–50 μg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membranes (30 volts) overnight. The blots were blocked in blocking-buffer (5% non-fat dry milk, Tris 20 mM, pH 7.6, NaCl 150 mM, 0.1% Tween-20) for 1 h at room temperature, followed by incubation with primary antibody overnight at 4°C. The membranes were washed and incubated for 60 min with a dilution of secondary antibody. Immunodetection was performed using the ECL detection kit (Amersham, UK). β-actin served as a loading control.

Catalase assay

The enzyme activity of catalase was measured using the TiSO₄ method [24], and the results are expressed as Units/mg of protein.

In vivo experiments

To generate subcutaneous tumors with K562 or BaF3 cells, female Balb/c nude mice (Harlan, The Netherlands) were injected with 10 × 10⁶ K562 or BaF3 cells diluted in a 50% ratio in Matrigel (BD Biosciences, San Jose, CA). When the tumor reached 200 mm³, mice were divided into two groups and treated with either saline or asc/men (1 g/kg, 10 mg/kg) solution injected intraperitoneally (i.p.) every other day. All solutions were extemporaneously prepared in saline and sterilized by filtration through a 0.2 μm filter (Millipore, Billerica, MA). Tumors were measured daily with a caliper and the volume was calculated using the following formula: (length x width² × π)/6.
Alternatively, Balb/c mice (Charles River, Wilmington, MA) were injected intravenously (tail vein) with \(1 \times 10^6\) BaF3 cells (either WT or T315I for Bcr-Abl), resulting in an aggressive malignancy resembling acute leukemia [25, 26]. Control mice were injected with saline. After 24 h, mice were divided into two groups and treated with either saline or asc/men (1 g/kg, 10 mg/kg) solution injected i.p. All solutions were extemporaneously prepared in water. Twelve days after tumor inoculation, mice were killed in order to avoid unnecessary suffering of the animals and total blood was collected to count peripheral white blood cells using a Sysmex K-1000 automated hematology analyzer (Sysmex, Kobe, Japan).

EPR measurements

The EPR spectra were acquired at room temperature using an EPR Elexys E540 System (Bruker, Rheinstetten, Germany) equipped with a X-Band EPR Super High Q cavity cylindrical resonator (ER 4122SHQE, 10 mm diameter) operating at \(\sim 9.5\) GHz. A flat cell for aqueous samples (ER 160 FC-Q, Bruker, Germany) was filled with 400 microliters of the solution and positioned in the resonator with its flat side perpendicular to the direction of the field. Spectra were recorded in complete medium or in medium supplemented with RBC (1% hematocrit) with various concentrations of ascorbate and menadione (for ascorbyl radical measurements) or \(\text{H}_2\text{O}_2\) (for hydroxyl radical measurements). The maximum microwave power level was 5 mW for ascorbyl detection (based on previous experiments [9]). Other parameters were as follows: Center field 3474 G (347.4 mT), sweep width 20 G (2 mT), modulation frequency 100 kHz, modulation amplitude 0.6 G (0.06 mT), time constant 40.96 ms, conversion time 10.24 ms, resolution 512 points, 10 scans. The hydroxyl radical spectrum was recorded using 50 mM DEPMPO added together with various concentrations of \(\text{H}_2\text{O}_2\). Parameters were the same as for ascorbyl radical, except for the following: Power 16.4 mW, modulation amplitude 1 G (0.1 mT), center field 3472 G (347.2 mT), sweep width 200 G.

All spectra were recorded 10 min after the addition of asc/men or \(\text{H}_2\text{O}_2\) into the medium.

Statistical analyses

Data were analyzed using an ANOVA test followed by a post-hoc Tukey test to determine the statistical significance among the different groups. When only two groups had to be compared, a t-test was used. Tumor volume evolution was analyzed using an ANOVA two-ways test. The level of significance was set at \(p \leq 0.05\) versus control (*\(= p \leq 0.05\); **\(= p \leq 0.01\); ***\(= p \leq 0.001\)). All analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

Results

In vitro effects of asc/men

Drug selectivity is a critical issue in any therapeutic approach. Indeed, the ultimate question for every compound expected to possess biological activity is its clinical relevance (i.e., efficacy and safety). In this context, three different cell types: a stable human CML cell line (K562), peripheral leukocytes derived from patients with CML, and peripheral leukocytes derived from healthy donors, were exposed to oxidative stress induced by asc/men. Figure 2a shows that asc/men was active against both types of CML cells but was less cytotoxic in normal leukocytes. This differential sensitivity may be explained by the fact that the expression and activity of catalase are markedly decreased in K562 cells compared to normal leukocytes (Fig. 2b). Moreover, Bcr-Abl stability is dependent on the chaperoning activity of Hsp90, and we showed that Hsp90 was cleaved when cells were exposed to asc/men (Fig. 2c), leading to Bcr-Abl degradation.

We then assessed whether asc/men could be active against cells with mutations in Bcr-Abl that render them resistant to standard anticancer treatment, namely imatinib. Figure 3a shows the effects of asc/men and imatinib on the proliferation of BaF3 cells transfected with either normal or mutated forms of Bcr-Abl. Imatinib was able to impair the proliferation of BaF3/Bcr-Abl-WT and BaF3/Bcr-Abl-T315I cells but had no effect on BaF3/Bcr-Abl-T315I cell proliferation. In contrast, asc/men impaired the proliferation of cells with or without a Bcr-Abl mutation. In addition to cell proliferation, Fig. 3b shows the effects of asc/men and imatinib on LDH leakage in Bcr-Abl WT-, E255K- and T315I-bearing cells. In each cell type, asc/men induced a considerable cell death after 24 h of incubation while imatinib induced cell death in WT and E255K cell lines but T315I cells remained totally resistant. Moreover, imatinib and asc/men should affect the activity of several phosphotyrosine proteins in Bcr-Abl-expressing cells. As expected, imatinib provoked strong dephosphorylation of such proteins in WT and E255K cells but no effect was observed in cells expressing the T315I mutation (Fig. 3c). In contrast, asc/men induced a strong decrease in the phosphorylation levels of phosphotyrosines whatever the cell line. Furthermore, asc/men induced rapid degradation of the Bcr-Abl protein irrespective of the cell line, whereas imatinib was...
unable to modify the amount of Bcr-Abl protein (Fig. 3d).
Since asc/men induces cleavage of Hsp90 with subsequent
disruption of its chaperone activity, which leads to the
degradation of its client proteins ([13], and Fig. 2c), we
explored whether the same profile could be observed in the
three transfected BaF3 cell lines. Indeed, Fig. 3e shows that
Hsp90 was cleaved in all asc/men-treated cells, but imatinib
did not affect the integrity of the Hsp90 protein.

In vivo effects of asc/men

We then decided to test whether the in vitro inhibitory effects
of asc/men are also observed under in vivo conditions.
Treatment of mice with asc/men (1 g/kg, 10 mg/kg, i.p.)
significantly reduced the growth rate of K562 tumors
compared to animals that received i.p. saline (Fig. 4a). At
day 21, control animals were killed to avoid unnecessary
suffering. At that time, the mean tumor volume was
420 mm$^3$, while in asc/men treated-mice tumor volume was
significantly less, by about 70\% (120 mm$^3$). Treated-mice
were kept alive and treated for an additional 10 days, and
then killed. The mean tumor volume at the moment of
sacrifice (day 31) was about 300 mm$^3$, a value still less than
the tumor volume in control mice at day 21.

Secondly, given the key role played by the Bcr-Abl
oncogene in the survival of transformed cells, and the cell
resistance to standard chemotherapy that is induced by Bcr-
Abl mutations, we tested the effect of asc/men in a model of
BaF3/Bcr-Abl-T315I cells growing as solid tumors.
Figure 4b shows the effect of repeated asc/men doses on
the proliferation of BaF3/Bcr-Abl-T315I cells. Treatment of
mice with asc/men significantly reduced the growth rate of
tumors compared to animals receiving i.p. saline. Control
animals were killed 17 days after cell injection, a time at
which the mean volume of the tumors was about
2400 mm$^3$. At the same time, in asc/men-treated animals
the mean tumor volume was about 480 mm$^3$, a value which
represents 20\% of the tumor volume in control untreated
animals. Treated animals were sacrificed 25 days after cell
injection.

Finally, we decided to test whether asc/men could
reduce the proliferation of BaF3 cells expressing either
wild-type (WT) or mutated Bcr-Abl in the blood of mice.
Figure 4c shows that asc/men treatment was not able to

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**Fig. 2** In vitro effects of asc/men. a Cytotoxicity of asc/men (2 mM/
10 $\mu$M) in several in vitro models: K562 cells, peripheral blood
leukocytes from CML patients and peripheral blood leukocytes from
healthy donors. Cells were prepared as described in the material and
methods section and incubated for 24 h with (Asc/Men 24 h) or
without (Ctrl) asc/men. Cytotoxicity was then assayed by measuring
LDH leakage as described in the material and methods section.
Results are mean±SEM of at least three independent experiments
with samples coming from different donors. Student t-tests were performed
to analyze the data. b Catalase expression (left) and activity (right) in
K562 cells compared to peripheral blood leukocytes from healthy
donors. Catalase expression from six different samples was assayed by
Western blot. Catalase activity was measured as described in the
material and methods section. Results are mean ± SEM of three
independent experiments with samples coming from different donors.
A Student t-test was performed to analyze the data. c Bcr-Abl and
Hsp90 expression were assayed in K562 cells by Western blotting.
Blots are representative of at least three independent experiments.
impair the proliferation of BaF3/Bcr-Abl cells, irrespective of the Bcr-Abl mutation. One explanation for this lack of effect could be that RBC can remove H$_2$O$_2$ from their environment and protect cells from H$_2$O$_2$-induced damages [27–30]. Since asc/men cytotoxicity is caused by oxidative stress with H$_2$O$_2$ as main oxidizing agent [4, 8, 9], we hypothesized that RBC detoxify H$_2$O$_2$ and protect cells from asc/men. Indeed, Fig. 5a shows that the addition of RBC to the medium completely suppressed the asc/men-induced cytotoxicity. This suppression was likely due to intracellular antioxidant defences of the RBC since the protective effect remained unchanged when lysed RBC were added to the medium. However, when RBC lysate was boiled for 5 min before adding to the culture medium, the protective effect decreased, indicating that the protective elements were most likely enzymatic. In addition, Fig. 5b shows that RBC decreased by 85% the ascorbyl radical formed during asc/men redox cycling. Since hydroxyl radical cannot be detected in the presence of ascorbate, we mimicked the effects of asc/men by using...
H$_2$O$_2$ to provoke the death of BaF3/Bcr-Abl cells. As expected, the addition of RBC totally suppressed H$_2$O$_2$-induced cytotoxicity (Fig. 5c) and strongly decreased hydroxyl radical formation (Fig. 5d).

**Discussion**

Oxidative stress induced by asc/men leads to cell death in several cancer cell lines [4, 8, 9, 11, 31] because these cells are highly sensitive to treatments that interfere with the maintenance of redox homeostasis [32, 33]. The rationale for this effect is the existence of a differential redox control of proliferation and viability in non-transformed versus malignant cells. Indeed, cancer cells usually exhibit high levels of ROS, which stimulate cell proliferation and promote genetic instability [5]. These constitutively high levels of cellular oxidative stress and dependence on ROS signalling represent a redox vulnerability of malignancies that can be targeted by chemotherapeutic interventions using redox modulators. This vulnerability is further enhanced by the fact that cancer cells are deficient in antioxidant enzymes [3, 4, 14], rendering them even more sensitive to oxidative stress.

In this study, we showed that asc/men kills cancer cells derived from human stable cell lines or from patients. Normal leukocytes from healthy donors were less sensitive to asc/men, probably due to their normal antioxidant status. We also showed that K562 cells are deficient in catalase activity as compared to normal leukocytes, so that asc/men induces cell death likely via the generation of H$_2$O$_2$. Previous work in our laboratory has shown that the toxicity of asc/men in several cancer cell lines is likely the consequence of an oxidative stress that impairs multiple intracellular targets such as glycolysis [11], calcium homeostasis [12], and protein chaperone activity [13]. Indeed, we found that oxidative stress leads to Hsp90 cleavage with a subsequent loss of its chaperone activity and degradation of several Hsp90 client proteins. Since the stability of Bcr-Abl (and other oncoproteins) is dependent on the chaperoning activity of Hsp90 [23], and given the critical role of this protein in cell survival, we looked for the effect of oxidative stress in cells expressing either the wild type or mutated forms of the protein. Indeed, we observed that survival and cell proliferation in each Bcr-Abl-bearing cell line were strongly affected by asc/men, irrespective of the type of Bcr-Abl mutation. Imatinib was also able to induce cell death of BaF3/Bcr-Abl-WT and BaF3/Bcr-Abl-T315I-bearing cell lines but the BaF3/Bcr-Abl-T315I-bearing cell lines were totally resistant. It should be noted that asc/men provoked the degradation of the three Bcr-Abl proteins. Moreover, the degradation of Bcr-Abl causes a loss of tyrosine kinase activity and consequently...
significantly decreased the levels of phosphotyrosine in the three cell lines. These processes appear to be the consequence of asc/men-mediated Hsp90 cleavage and not a direct effect on these proteins. As expected, imatinib decreased the phosphorylation of proteins in BaF3/Bcr-Abl-WT and BaF3/Bcr-Abl-E255K cells but was unable to inhibit Bcr-Abl-mediated phosphorylation in BaF3/Bcr-Abl-T315I cells.

When K562 and BaF3/Bcr-Abl-T315I cells were implanted in mice, asc/men strongly delayed the cell proliferation. Nevertheless, asc/men did not affect the proliferation of BaF3/Bcr-Abl cells when injected in blood of mice. Since asc/men was administered in the same manner (i.p. injections) in subcutaneous models, pharmacokinetic differences cannot explain this difference of effects. The most likely explanation is that RBC detoxify H$_2$O$_2$ and protect cells and tissues from the damage it induces [27–29]. In the particular case of cancer cells, Chen et al. report that RBC completely protect lymphoma cells from H$_2$O$_2$ generated by pharmacologic concentrations of ascorbate [30]. In the conditions in our study, both asc/men- and H$_2$O$_2$-mediated toxicities were prevented by RBC, suggesting that the lack of effect of asc/men in the blood was dependent on the antioxidant activity of RBC.

In conclusion, our results show that asc/men has promising in vitro and in vivo effects. Indeed, for cells expressing the oncogenic protein Bcr-Abl (or other Hsp90 client proteins), asc/men, by inducing oxidative cleavage of this protein chaperone, may kill cells irrespective of mutations in Bcr-Abl. Since peripheral blood leukocytes are less sensitive to asc/men than cancer cells, likely because of differences in antioxidant enzymes expression,
this makes asc/men a non-toxic possible adjuvant for chemotherapy.

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