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Enhancement of quinone redox cycling by ascorbate induces a caspase-3 independent cell death in human leukaemia cells. An *in vitro* comparative study

JULIEN VERRAX[1,†], MARIANNE DELVAUX[1], NELSON BEGHEIN[2], HENRYK TAPER[1], BERNARD GALLEZ[2], & PEDRO BUC CALDERON

[1]*Unité de Pharmacocinétique, Métabolisme, Nutrition et Toxicologie, Département des sciences pharmaceutiques, Université Catholique de Louvain, Brussels, Belgium, and* [2]*Unité de Chimie pharmaceutique et de radiopharmacie, Unité de Résonance magnétique biomédicale, Département des sciences pharmaceutiques, Université Catholique de Louvain, Brussels, Belgium*

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Abstract

Since the higher redox potential of quinone molecules has been correlated with enhanced cellular deleterious effects, we studied the ability of the association of ascorbate with several quinones derivatives (having different redox potentials) to cause cell death in K562 human leukaemia cell line. The rationale is that the reduction of quinone by ascorbate should be dependent of the quinone half-redox potential thus determining if reactive oxygen species (ROS) are formed or not, leading ultimately to cell death or cell survival. Among different ROS that may be formed during redox cycling between ascorbate and the quinone, the use of different antioxidant compounds (mannitol, desferal, *N*-acetylcysteine, catalase and superoxide dismutase) led to support H_2O_2 as the main oxidizing agent. We observed that standard redox potentials, oxygen uptake, free ascorbyl radical formation and cell survival were linked. The oxidative stress induced by the mixture of ascorbate and the different quinones decreases cellular contents of ATP and GSH while caspase-3-like activity remains unchanged. Again, we observed that quinones having higher values of half-redox potential provoke a severe depletion of ATP and GSH when they were associated with ascorbate. Such a drop in ATP content may explain the lack of activation of caspase-3. In conclusion, our results indicate that the cytotoxicity of the association quinone/ascorbate on K562 cancer cells may be predicted on the basis of half-redox potentials of quinones.

Keywords: *Ascorbate, quinone, redox cycling, reactive oxygen species, cancer*

Introduction

The association of ascorbate (vitamin C) and menadione (vitamin K_3) has been shown to be an effective chemosensitizer and radiosensitizer that induces little systemic or major organ pathology in TLT tumor-bearing mice [1,2]. Several reports have been published concerning the synergistic antitumor activity of the association of these two compounds (vitamin C and vitamin K_3) against a wide variety of human tumor cell lines [3–8] but the type of cell death is still a matter of controversy. Indeed, it has been recently suggested that the association of vitamins C and K_3 is killing cancer cells by autoschizis, a new type of cell death showing both apoptotic and necrotic morphologic characteristics [8–11].

Such an antitumor activity has been attributed to redox cycling of ascorbate and menadione and the possible generation of peroxides and other reactive

Correspondence: P. Buc Calderon, Unité PMNT 7369, 73, avenue E. Mounier, 1200 Bruxelles, Belgium. Tel: 32 2 764 7366. Fax: 32 2 764 7359. E-mail: calderon@pmnt.ucl.ac.be

[†]Julien Verrax is a FRIA recipient.

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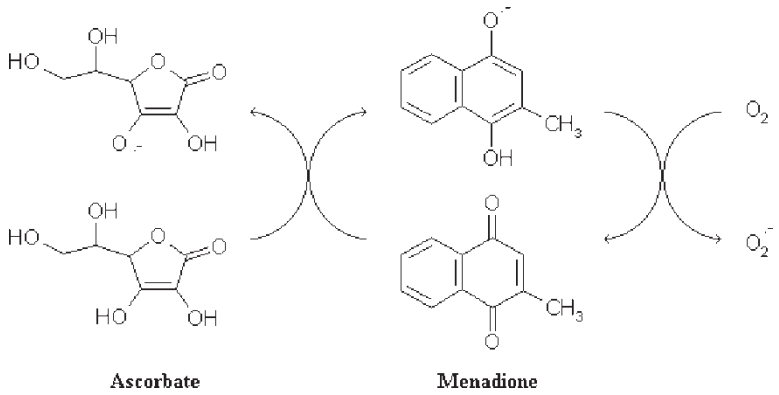

Figure 1. Redox cycling between ascorbate and menadione.

oxygen species (ROS). Indeed, H_2O_2 may be generated during the futile redox-cycling of the quinone moiety of menadione. In solution, menadione is non-enzymatically reduced by ascorbate to form semidehydroascorbate and the semiquinone free radical (Figure 1). Such a semiquinone is rapidly reoxidized to its quinone form by molecular oxygen [12] thus generating ROS such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$). In addition to redox cycling, menadione, a naphtoquinone with a double bond α to a keto group, can undergo a Michael addition to form adducts with sulfhydryls and primary amines leading to cell injury and cell death [13,14]. To discriminate which of both pathways (redox cycling or covalent binding) are involved in the cytotoxicity, DMNQ (2,3-dimethoxy-naphtoquinone), a vitamin K_3 analog without arylation sites, was used. The association of ascorbate with DMNQ instead of menadione, produced the same profile of cytotoxicity as observed with the association ascorbate/menadione, underlining the key role of the redox cycling pathway [8].

Since the higher redox potential of quinone molecules has been correlated with enhanced DNA strand breaks [15], in the current study we report on the ability of the association of ascorbate with several quinones derivatives (having different redox potentials) to cause cell death in K562 cells, a chronic myeloid leukaemia cell line (characterized by the Philadelphia chromosome). Briefly, possible mechanisms involved in such a cell death caused by this association (ascorbate/quinone) were investigated as well as its capacity to generate ROS. Additional markers to evaluate the cytotoxicity by ascorbate/quinone include both GSH and ATP contents, and caspase-3 activation.

Materials and methods

Cell line and cells culture conditions

The K562 cell line was a gift of Dr F. Brasseur (LICR-Brussels). They were cultured in DMEM/F12 (Dulbecco's modified eagle medium, Gibco) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml). The cultures were maintained at a density of $1-2 \times 10^5$ cells/ml. The medium was changed at 48–72 h intervals. All cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere with 100% humidity. The human hematopoietic stem cells were obtained from the Hematological Center of our University (School of Public Health). Mononuclear cells were isolated by centrifuging blood under Ficoll gradient, and incubated under the same condition as previously described for K562 cells.

Chemicals

Lawsone, 1,4-naphtoquinone, dichlone, anthraquinone, coenzyme Q_0, menadione bisulfite (vitamin K_3), sodium ascorbate (vitamin C), catalase, superoxide dismutase, mannitol, *N*-acetylcysteine, thiobarbituric acid and dimethylsulfoxide (DMSO), were purchased from Sigma (St. Louis, MO, USA). Ficoll-Paque was purchased from Pharmacia-Biotech AB (Uppsala, Sweden). Desferal was purchased from Ciba Pharmaceutical Co (Summit, NJ). The Caspase 3 inhibitor, Z-DEVD-FMK, was purchased from Calbiochem (San Diego, CA). All other chemicals were ACS reagent grade.

Assays with cell-free systems

Most of the experimental procedures utilized to determine the formation of ROS are based on redox reactions (for instance, NBT and ferricytochrome *c* reduction as well as adrenaline autoxidation to determine superoxide anion). In addition, the use of spin-traps is not adequate under our experimental conditions since spin-adducts will be reduced to hydroxylamine by ascorbate. Since the mixture we employed contain both oxidants and reducing agents, we decided to evaluate the redox cycling between ascorbate and the respective quinone by measuring

the formation of ascorbyl radical and the reduction of molecular oxygen by the Clark electrode.

Oxygen uptake from mixtures of ascorbate and the respective quinone compound dissolved in 5 ml of well oxygenated phosphate buffer (0.05 M) was recorded by using a Clark electrode, the YSI Model 530 Biological Oxygen Monitor (Yellow Springs Instrument Co, Yellow Springs, OH). All the measures were made at 37°.

The electron paramagnetic resonance (EPR) spectra were obtained using a Bruker model EMX EPR spectrometer equipped with a variable temperature controller BVT-3000. All measurements were made at 37°. The experimental parameters (signal calibration and signal-to-noise ratio) were optimized for each sample. The parameter settings were frequency, 9.785 GHz; microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 G; and scan time, 20.9 s. The recording of EPR spectra was started 1 min after inserting a sample into the cavity.

Cellular assays

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue [16], both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as a ratio of released activity to the total activity.

ATP content was determined by using the biolumiscence Boehringer kit and the results are expressed as nmol ATP/mg proteins. The amount of protein content was determined by the method of Lowry [17] using BSA as reference.

Reduced glutathione (GSH) was measured according to the OPT method as reported by Cohn and Lyle [18]. The caspase-3 activity was monitored by cleavage of a specific peptide substrate, Asp-Glu-Val-Asp-AFC (DEVD-AFC) according to the procedure outlined in the instructions for the "FluorAce apopain assay" kit (Bio-Rad). Briefly, after incubation with the test compounds for 2, 4 and 6 h, the cells were washed twice with PBS, lysed and centrifuged. The supernatants were incubated with DEVD-AFC in the reaction buffer supplied with the kit. Substrate cleavage was determined kinetically at room temperature in a LS50B Luminescence Spectrometer (375 nm excitation, 510 nm emission) (Perkin Elmer, Urbana, IL).

Lipid peroxidation was assessed by measuring the formation of malondialdehyde (MDA)-like substances, namely the thiobarbituric-reacting substances (TBARS) method. Briefly, cell samples were washed twice with cold PBS before sonication. Cell samples (200 μl in PBS) were mixed with 1.5 ml of phosphoric acid 1%, and either 500 μl of thiobarbituric acid 0.6% (tests) or 500 μl of water (blanks). They were heated in a water bath for 1 h at 95°C and then allowed to cool. The colored reaction product was extracted with 2 ml *n*-butanol, and the difference of the absorbance at 535 and 520 nm was recorded. The results were expressed as nanomoles of MDA per milligram of protein.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Scheffé test for significant differences between means. For statistical comparison of results at a given time point, data were analyzed using Student's *t*-test. Statistical analysis of correlation was performed using Pearson Correlation test. The level of significance was set at $p < 0.05$.

Results

The effect of ascorbate (2 mM) either alone or combined with different concentrations of menadione (ranging from 2 to 20 μM) on the oxygen uptake in a free-cell system is shown in Figure 2A. As compared to rates observed with ascorbate alone (8.8 ± 0.2 nmol O_2/min) the addition of increased doses of menadione resulted in an enhanced oxygen uptake reaching rates of 15.7 ± 0.5, 22.1 ± 0.3 and 30.3 ± 0.6 nmol O_2/min for K_3 at 5, 10 and 20 μM, respectively. No difference in oxygen uptake was observed when ascorbate was combined to menadione at 2 μM (10.0 ± 0.9 nmol O_2/min). In a similar way, the K562 cell death was shown to be dependent of the menadione concentration (Figure 2B). Indeed, after 24 h incubation the association of ascorbate with menadione at either 2, 5, 10 and 20 μM, induced a LDH leakage of 32, 41, 50 and 67%, respectively, as compared to 10% observed in control conditions. Such a cytotoxicity was confirmed by dye exclusion (data not shown). Figure 2C shows the correlation ($r = 0.98$; $p < 0.01$) between cell death and oxygen uptake, suggesting that cell death increases as a function of ROS formation. Finally, the Figure 2D shows the formation of free ascorbyl radical when ascorbate (2 mM) is associated with different concentration of menadione. As previously observed with the oxygen uptake, the formation of ascorbyl radical is dependent of the concentration of menadione.

According to previous results obtained with other cell lines, such as TLT mouse hepatoma [19,25], human foreskin fibroblasts [5], and human oral cell lines [7], our results show that the association of ascorbate/menadione is mainly killing the transformed K562 cell line (Figure 3A) while it was devoid of cytotoxicity against the erythropoietic normal stem cell line where K562 is mostly derived. Regarding what reactive species are being formed during the redox cycling, Figure 3B shows that neither superoxide anion nor hydroxyl radical seem to play a major role in this cytotoxicity since the addition of superoxide dismutase, desferal or mannitol do not

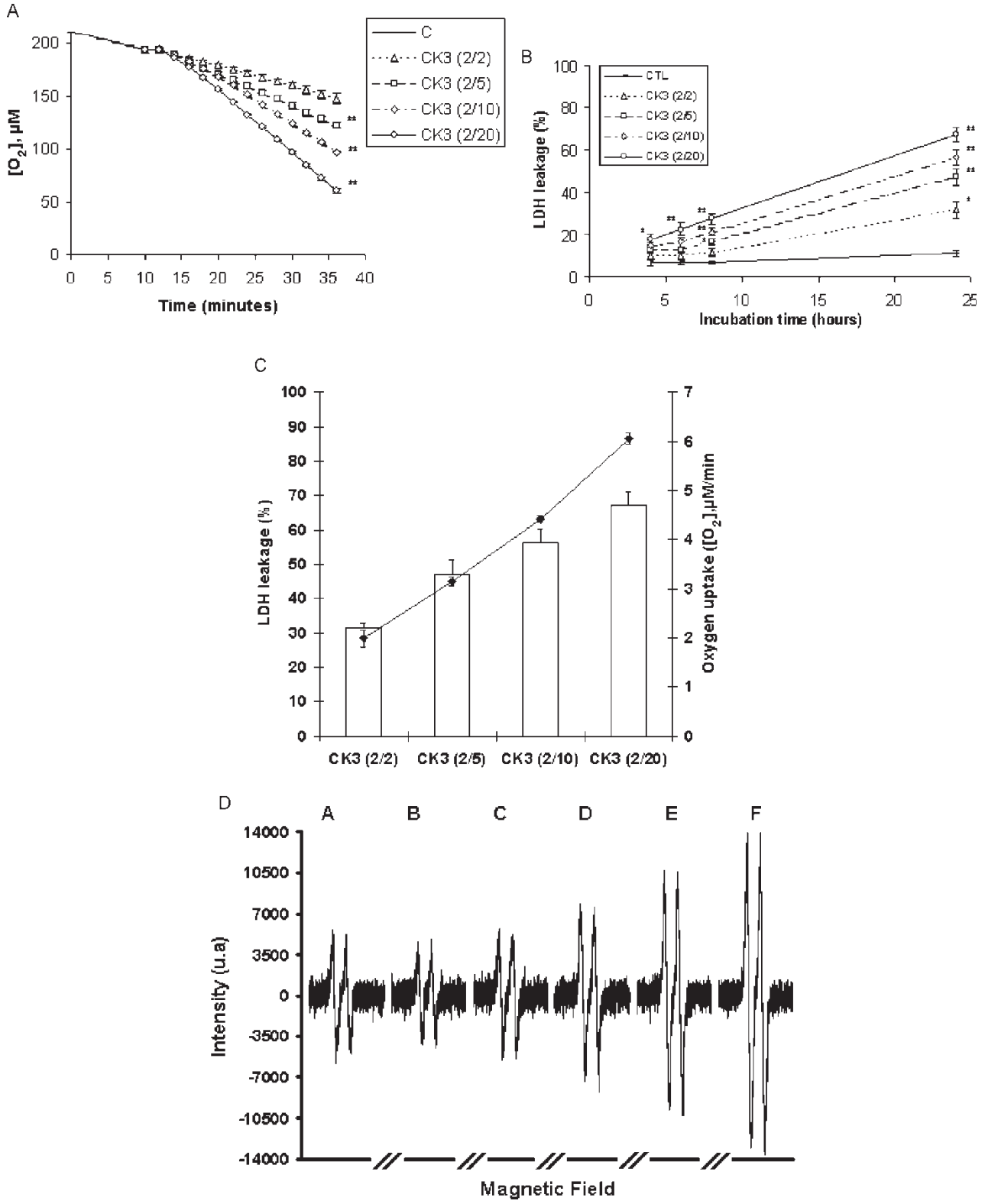

Figure 2. (A) Effect of different concentrations of menadione (2, 5, 10 and 20 µM) (vitamin K_3) on the oxygen uptake in the presence of 2 mM ascorbate (vitamin C). The results are mean values of three separate experiments ± SEM. $^{**}p < 0.01$ as compared with ascorbate alone. (B) Effect of different concentrations of menadione (2, 5, 10 and 20 µM) (vitamin K_3) on K562 cell death in the presence of 2 mM ascorbate (vitamin C). The results are mean values of three separate experiments ± SEM. $^{*}p < 0.05$ as compared with CTL (none vitamins); $^{**}p < 0.01$ as compared with CTL. (C) Correlation between oxygen uptake and cell survival. K562 cells were incubated in the presence of ascorbate (2 mM) and menadione (2, 5, 10 and 20 µM) for 24 h. The results are mean values of three separate experiments ± SEM. (D) Ascorbyl radical intensity in the presence of increasing doses of menadione. A: Ascorbate 2 mM; B: A + Menadione 1 µM; C: A + menadione 10 µM; D: A + menadione 100 µM; E: A + menadione 1 mM; F: A + menadione 10 mM. Typical EPR spectra out of three separate experiments are represented.

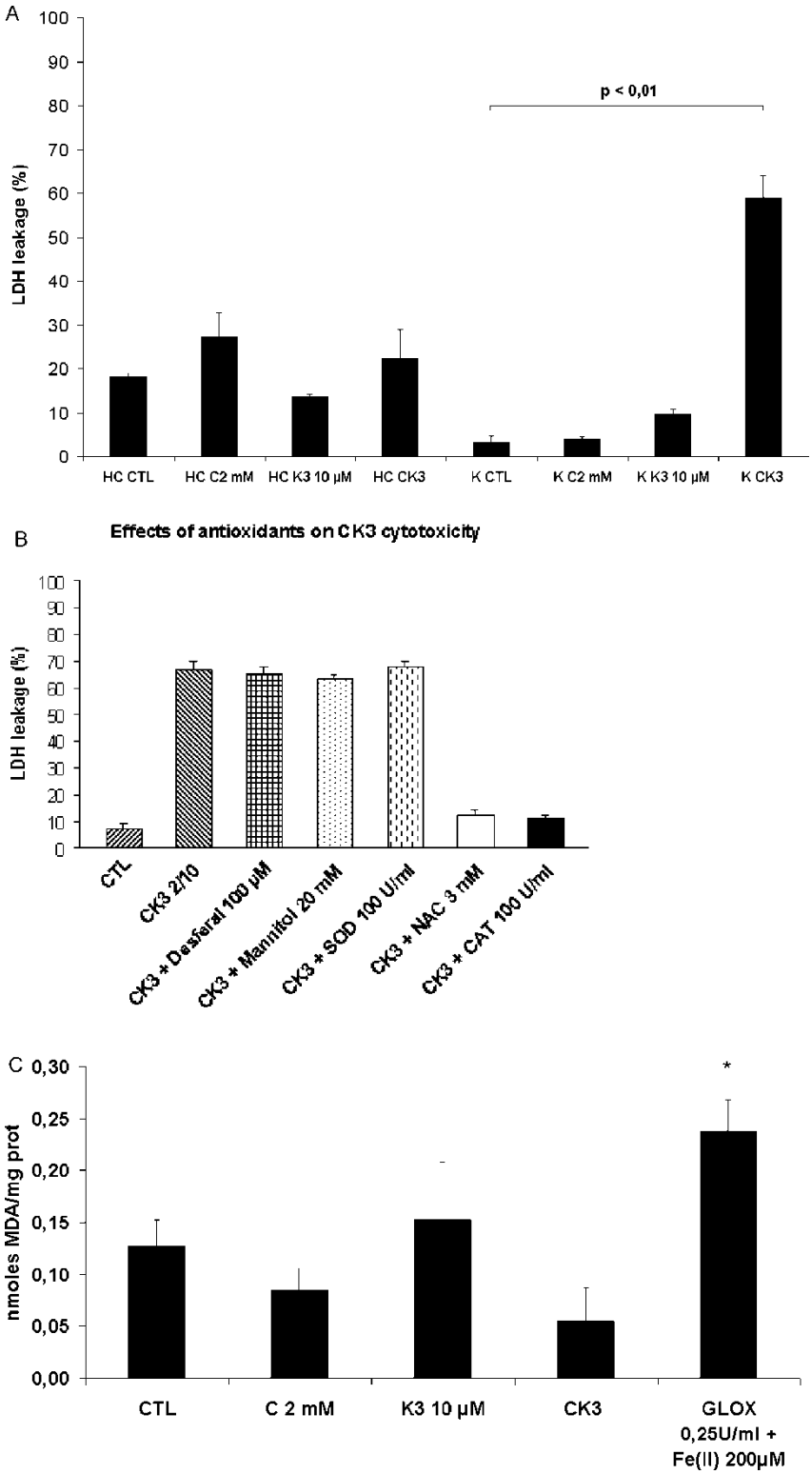

Figure 3. (A) Cytolytic effect of the association ascorbate/menadione towards healthy cells (HC = human hematopoietic stem cells) versus human leukaemia cells (K = K562). Human hematopoietic stem cells were obtained from two different donors. Cells were incubated for 24 h in the absence and in the presence of ascorbate (2 mM), menadione (10 µM) and a mixture of both compounds. Each incubation was performed in triplicate. The results are mean values ± SEM. (B) Effect of different antioxidants on the cytotoxicity of the association ascorbate/menadione. K562 cells were incubated for 24 h. Ascorbate was used at 2 mM, menadione at 10 µM, mannitol at 20 mM, desferal at 100 µM, *N*-acetyl-cysteine (NAC) at 3 mM and superoxide dismutase and catalase both at 100 U/ml. The results are mean values of three separate experiments ± SEM. (C) Effect of vitamins on lipid peroxidation. K562 cells were incubated for 6 h. Ascorbate was used at 2 mM, menadione at 10 µM. Glucose oxidase (GLOX, 0.25 U/ml) plus iron ($FeCl_2$; 200 µM) was used as positive control. The results are mean values of three separate experiments ± SEM. $^*p < 0.05$ as compared with control.

prevent the cell lysis. However, H_2O_2 appears to be the main oxidizing agent generated during the redox cycling between ascorbate and menadione since the addition of *N*-acetylcysteine or catalase, both involved in hydrogen peroxide detoxification, completely suppress the cytotoxicity. Moreover, it was further shown that lipid peroxidation, as measured by the formation of TBARS, was not involved in such a cell death since control values were 0.13 nmol MDA/mg of protein as compared to 0.05 nmol MDA/mg of protein observed in the presence of ascorbate/menadione (Figure 3C).

The redox cycling between ascorbate and menadione thus generating H_2O_2 appears then as a key element in their cytotoxicity. Therefore, the reduction of quinone by ascorbate should be dependent of the quinone redox potential thus determining if ROS are formed or not. Indeed, Table I shows the values obtained for standard redox potentials, oxygen uptake and the formation of free ascorbyl radical radical when ascorbate is associated with different quinones at 10 μM. For instance lawsone ($E_{1/2}$ of −415 mV), menadione ($E_{1/2}$ of −203 mV) and dichlone ($E_{1/2}$ of −36 mV) are consuming 9.8, 22.1 and more than 500 nmol O_2/min respectively, when they were associated to ascorbate. Furthermore, the association of ascorbate with quinones having a low half-redox potential (anthraquinone and lawsone) did not result in an enhanced formation of ascorbyl radical. Among these effective compounds, 1–4 naphtoquinone, coenzyme Q_0 and dichlone cause a significant increase in the amount of ascorbyl radical (plus 305, 374 and 247% respectively) but only a slight increase (16%) is observed with menadione. Figure 4 shows that the cell lysis, however, exhibited a different profile: the values of LDH leakage by mixtures of ascorbate to quinones that did not induce an oxygen uptake and have low redox potential (−445 and −415 mV), were similar to that observed in untreated control cells (around 10%). Although, the other mixtures induced an $E_{1/2}$-dependent oxygen uptake, the LDH values were rather similar (around 60%) but statistically higher than untreated control cells. The cell lysis under those conditions was totally independent from either the redox potential or the oxygen uptake suggesting that ROS while being produced at different rates, they are reaching a threshold from which the cell lysis became independent of their concentrations. The quinones by themselves were devoid of any cytotoxic effect, at least at the concentration that were employed.

Table II shows the effect of the association of ascorbate (2 mM) with 10 μM of quinone on cellular contents of ATP and GSH and on caspase-3-like activity. Once again, it was observed severe deleterious effects on ATP and GSH contents when a quinone with a high value of half-redox potential is associated with ascorbate. Indeed, with the exception of lawsone and anthraquinone, ATP and GSH were depleted by about 60 and 30% after 8 h of incubation. Nevertheless, as previously observed with cell lysis, the association of ascorbate with menadione ($E_{1/2}$ of −203 mV) shows the same profile as that of ascorbate with dichlone ($E_{1/2}$ of −36 mV), reinforcing the idea of a threshold in the formation of ROS.

Regarding the effect of the association of ascorbate with different quinones on DEVDase activity, we confirmed previous results showing that the association of ascorbate/menadione is killing cancer cells by a caspase-3-like independent way [19]. Indeed, none of the associations of quinones with ascorbate was able to induce the cleavage of the fluorogenic peptide indicating the absence of a caspase-3-like activity. This absence of caspase-3-like activity was further confirmed by immunoblotting showing that neither processing of procaspase-3 nor PARP cleavage occurs (data not shown). This lack of effect was completely independent of the half-redox potential of the quinones but geranylgeraniol was able to induce by about 5 times such a caspase-3-like activity. The addition of a caspase-3 inhibitor, namely the fluoromethylketone Z-DEVD-FMK (7.5 μM), abolished completely such an activity.

Discussion

Several reports have shown that cell death by ascorbate [20,21] or by menadione [22–24] is related with loss of redox homeostasis. Moreover, we have recently reported that cell death is only observed when ascorbate and menadione were added in combination whereas when added by separate they are deprived of cytotoxicity [25]. Several human cell lines have been shown to be extremely sensitive to the association of ascorbate/menadione [3,5,7,8]. In agreement with previous reports showing that cancer cells such as human oral squamous cell carcinoma (HSC-2, HSC-3) were more sensitive to the association of menadione/ascorbate as compared to normal cells [7], we also show that ascorbate/menadione kills more efficiently cancer cells than the precursor normal cells (Figure 3A). This rather selective effect on cancer cells is likely due to their extreme sensitivity to oxidative stress, most probably because their low activities of antioxidant enzymes [5,26,27]. In addition, ascorbate is preferentially taken up by cancer cells as compared to normal cells [28,29] a fact which facilitate the redox cycling between the two vitamins.

The selective activity of the association ascorbate/menadione against cancer cells raised the question about the mechanisms conditioning the cell death. We suggest that only the quinones that have redox potentials between −250 and +50 mV are able to oxidize ascorbate thus generating a redox cycling and the formation of ROS, mainly H_2O_2. Our results are in agreement with those reported by Roginsky et al. [30,31] and O'Brien [32] since, neither anthraquinone

Table I. Effect of the association of ascorbate with quinone on radical ascorbyl intensity and oxygen uptake.

Compounds	Redox potentials (mV) ($Q/Q^{\cdot -}$)	Oxygen uptake [O_2] μM/min	Ascorbyl radical intensity (%)
Ascorbate		1.76 ± 0.04	100 ± 1.3
Ascorbate + Anthraquinone	−445	1.78 ± 0.12	104.1 ± 12.2
Ascorbate + Lawsone	−415	1.96 ± 0.08	101.3 ± 10.8
Ascorbate + Menadione	−203	4.42 ± 0.06**	116.4 ± 10.3
Ascorbate + 1-4 Naphtoquinone	−140	94.3 ± 1.04**	404.9 ± 21.8**
Ascorbate + Coenzyme Q_0	−110	97.58 ± 0.7**	474.1 ± 40.5**
Ascorbate + Dichlone	−36	100.26 ± 1.06**	346.9 ± 45.1**

Oxygen uptake was recorded at 37°C in well oxygenated phosphate buffer (50 mM, pH 7.4) using a Clark electrode as described under "Materials and methods" section. Ascorbyl radical intensity was recorded at 37°C in phosphate buffer (50 mM, pH 7.4) using a Bruker model EMX EPR spectrometer as described under "Materials and methods" section. Ascorbate was used at 2 mM and quinones at 10 μM. The results are mean values of three separate experiments ± SEM. **$p < 0.01$ as compared with ascorbate alone (control value). (Ascorbate half redox potential: +282 mV).

nor lawsone, both having a half-redox potential lower than −250 mV, were able to oxidize ascorbate.

Our hypothesis on the critical role of redox cycling between ascorbate and quinones was confirmed by measuring oxygen uptake and the formation of ascorbyl radical by EPR. Such studies indicate that half-redox potentials influence in a significant way the formation of ROS. Therefore, the resulting cytotoxicity of ascorbate and a given quinone may, in same extent, be predicted on the basis of the quinone standard redox potential. Cell death is then caused by the oxidative stress generated by the ROS formed during the redox cycling. Among such ROS, our present data and previous results [8,25], strongly suggest that H_2O_2 is the main oxidizing agent. Furthermore, lipid peroxidation, as measured by TBARS, was not involved in the mechanisms of cytotoxicity induced by the association of ascorbate/menadione. This result is in agreement with a previous report by Jamison et al. [11] who shows that in T24 cells (a human bladder carcinoma cell line) the process of lipid peroxidation induced by the association of ascorbate/menadione is more a consequence of cell death than a causative effect.

The oxidative stress induced by the mixture of ascorbate and the different quinones (as a function of their redox potential) decreases the levels of both GSH and ATP. Interestingly, oxidative stress should leads to a decrease in GSH than ATP levels, but it is rather the inverse that occurs. Such a paradox is only apparent because the main GSH consuming processes are depressed in cancer cells, which are strongly dependent on glycolysis in order to get the required energy. It seems that the strong ATP depletion induced by the association of ascorbate and some quinones, may reflect an impairment in the glycolytic pathway. In agreement with this, it has been reported that glycolytic flow was stopped and glyceraldehyde-3-phosphate dehydrogenase was inactivated when cells were incubated in the presence of H_2O_2 [33]. Such a drop in ATP content may explain the lack of activation of caspase-3, a hallmark of

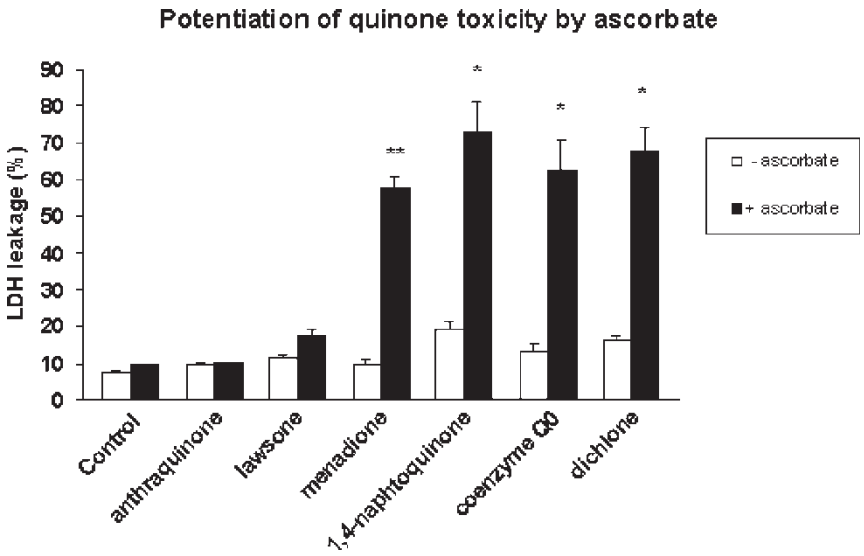

Figure 4. Cytolytic effect of quinone compounds in the absence or in the presence of ascorbate. K562 cells were incubated for 24 h in the presence of quinones (10 μM), with or without ascorbate (2 mM). The results are mean values of four separate experiments ± SEM. **$p < 0.01$ as compared with control.

Table II. Effect of the association ascorbate/quinone on ATP and GSH content and on caspase-3 activity.

Compounds	ATP content (nmol/mg proteins)	GSH content (nmol/mg proteins)	Caspase-3 activity (mUnits/mg proteins)
None (CTL)	14.7 ± 0.4	30.4 ± 4.2	0.9 ± 0.1
Ascorbate + Anthraquinone	10.0 ± 0.4**	24.0 ± 5.0	0.7 ± 0.1
Ascorbate + Lawsone	12.0 ± 0.5*	23.1 ± 5.7	0.8 ± 0.1
Ascorbate + Menadione	4.3 ± 0.7**	14.8 ± 1.2*	0.7 ± 0.0
Ascorbate + 1-4 Naphtoquinone	7.5 ± 0.6**	19.1 ± 3.4*	0.3 ± 0.0
Ascorbate + Coenzyme Q_0	7.8 ± 0.6**	17.0 ± 4.1*	0.7 ± 0.0
Ascorbate + Dichlone	5.0 ± 0.8**	16.1 ± 3.4*	0.7 ± 0.0
Geranylgeranyol			4.3 ± 0.3**
Geranylgeranyol + Z-DEVD-FMK			0.02 ± 0.01

Cells were incubated for 8 h in the absence or in the presence of quinones (10 μM) and ascorbate (2 mM) either alone or in combination. Aliquots of cell suspension were taken and ATP and GSH contents were measured as described under "Materials and methods" section. Caspase-3 activity was measured after 3 h of incubation. Geranylgeranyol was used at 100 μM as a positive control for caspase-3 activity. Z-DEVD-FMK is a peptide inhibitor of caspase-3, it was used at 7.5 μM to control the specificity of the reaction. Results represent the mean values ± SEM from at least 3 experiments. *$p < 0.05$ as compared with control values (untreated conditions); **$p < 0.01$ as compared with control values.

apoptosis [34,35], we observed in both K562 (Table II) and TLT cells [19], since the recruitment and processing of caspase-9 by the apoptosome complex requires ATP [36]. Another possibility is that the critical cysteine residue, within the QACRG motif in the caspase catalytic site [37,38], may be oxidized by H_2O_2 rendering the enzyme inactive. On the basis of previous biochemical data and morphological observations, it appears that the association of ascorbate and quinones is redirecting cells to another form of cell death, which is neither apoptosis nor necrosis, which some authors have named "autoschizis" [8–11,19,25]. In conclusion, our results indicate that on the basis of half-redox potentials of quinones it is possible to predict the cytotoxicity of the association quinone/ascorbate on cancer cells. Such a combination could represent a new non-toxic auxiliary cancer therapy without presenting any supplementary risk for the patients.

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