Apoptosis 2004; 9: 223–233

Ascorbate potentiates the cytotoxicity of menadione leading to an oxidative stress that kills cancer cells by a non-apoptotic caspase-3 independent form of cell death

Julien Verrax*, Julie Cadrobbi, Carole Marques, Henryk Taper, Yvette Habraken, Jacques Piette and Pedro Buc Calderon

Unité de Pharmacocinétique, Métabolisme, Nutrition et Toxicologie, Département des Sciences Pharmaceutiques, Université Catholique de Louvain, Belgium (V. Julien, C. Julie, M. Carole, T. Henryk, B. C. Pedro); Laboratoire de Virologie, Université de Liége, Belgium (H. Yvette, P. Jacques)

Hepatocarcinoma cells (TLT) were incubated in the presence of ascorbate and menadione, either alone or in combination. Cell death was only observed when such compounds were added simultaneously, most probably due to hydrogen peroxide (H_2O_2) generated by ascorbate-driven menadione redox cycling. TLT cells were particularly sensitive to such an oxidative stress due to its poor antioxidant status. DNA strand breaks were induced by this association but this process did not correspond to oligosomal DNA fragmentation (a hallmark of cell death by apoptosis). Neither caspase-3-like DEVDase activity, nor processing of procaspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP) were observed in the presence of ascorbate and menadione. Cell death induced by such an association was actively dependent on protein phosphorylation since it was totally prevented by preincubating cells with sodium orthovanadate, a tyrosine phosphatase inhibitor. Finally, while H_2O_2, when administered as a bolus, strongly enhances a constitutive basal NF-κB activity in TLT cells, their incubation in the presence of ascorbate and menadione results in a total abolition of such a constitutive activity.

Keywords: ascorbate; menadione; oxidative stress; autoschizis; hepatocarcinoma cells.

Abbreviations: TLT: Transplantable Liver Tumor; LDH: lactate deshydrogenase.

Introduction

A large body of evidence supports the idea that loss of redox homeostasis (oxidative stress) induced by menadione leads to cell death by either necrosis or apoptosis, depending upon the dose and the exposure time of the oxidizing agent, and the cell type as well.[1–3] Such a cytotoxicity is largely enhanced when menadione is associated with ascorbate in a ratio of 1/100.[4] The rationale of this approach was first based in the findings that reactivation of both acid and alkaline DNases by ascorbate and menadione respectively, led to cancer cell death and tumour regression.[5–7] Secondly, the redox cycling between ascorbate and menadione generating hydrogen peroxide (H_2O_2) was thought to explain (at least partly) such a cytotoxicity.

The molecular mechanisms of this cell demise induced by the association of ascorbate and menadione have not been completely elucidated, but their *in vivo* administration produced the following effects: cancer growth inhibition in TLT-bearig mice, a murine ascites Transplantable Liver Tumour[8]; selective potentiation of tumour chemotherapy[9]; sensitization of tumours resistant to some drugs[10]; and potentiation of radiotherapy.[11] Interestingly, such a co-administration did not enhance the general toxicity that accompanies cancer chemotherapy, suggesting that increase in animal life span was due to cancer cell death without secondary inflammatory reactions. Up to now, several reports have been published concerning the antitumour activity of the association of ascorbate and menadione against a wide variety of human tumour cell lines.[4,12–16] More recently, it has been suggested that this association kills cancer cells by autoschizis, a new type of cell death showing both apoptotic and necrotic morphologic characteristics.[15,17–19] In the current study we have sought to further examine the mechanisms by which the association of ascorbate and menadione cause cell death in TLT cells, a murine hepatoma cell line. Several issues concerning the cytotoxicity, the profile of cell death and the possible mechanisms underlying their mediated effects were studied. We found that cell death was the result of

*Fria research recipient.

Correspondence to: B. C. Pedro, Unité PMNT, UCL 7369, Avenue E. Mounier 73, 1200 Bruxelles, Belgium. Tel: 32-2-764.73.66; Fax: 32-2-764.73.59; e-mail: calderon@pmnt.ucl.ac.be

a synergy between ascorbate and menadione due to their redox cycling generating H_2O_2, it was not dependent of caspase-3 activation, it involved tyrosine phosphorylation and it did not require NF-κB activation.

Materials and methods

Cell line and cells culture conditions

The murine hepatoma cell line, Transplantable Liver Tumor (TLT) were 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 $\times$ 10^5 cells per 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. Actinomycin D was used as a positive control.[20]

Chemicals

Menadione bisulfite, sodium ascorbate, phenylmethylsulfonylfluoride (PMSF), dimethylsulfoxide (DMSO), actinomycin D, and deoxycholic acid (DOC) were purchased from Sigma (St Louis, MO). Hybond membranes were purchased from Amersham (Arlington Hts, IL). The Caspase 3 inhibitor, Ac-DEVD-FMK, was purchased from Calbiochem (San Diego, CA). The Williams'E culture medium was from GIBCO (Grand Island, NY). Polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used for the determination of procaspase-3 (dilution 1/200) and PARP (dilution 1:200). All other chemicals were ACS reagent grade.

Assays

Immunoblotting of the protein fractions. At the indicated times, cells were washed twice with ice-cold potassium phosphate buffered saline (PBS) and then resuspended in a PBS lysis buffer containing 0.1% phenylmethylsulfonyl fluoride, 0.1% Tween-20, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate (SDS). The samples were kept on ice for 20 min and then centrifuged at 13000 $\times$ *g* for 10 min at 4°C. The proteins were subjected to SDS-PAGE (6–15% separating gel) followed by electroblot to PVDF or nitrocellulose membranes. The membranes were blocked 1 h in TBS buffer (pH 7.4) containing 5% powdered milk protein followed by an incubation with diluted antibodies in a fresh solution of powdered milk protein (1% w/v) in TBS buffer. The membrane was washed and incubated for 60 min with a dilution of secondary antibody coupled to alkaline phosphatase. Immunodetection was performed by colour development with NBT/BCIP.

DNA fragmentation studies. DNA fragmentation was determined by two techniques. In the first the DNA was extracted by either the Wizard Genomic DNA Purification system (Promega, Madison, WI) or the TACS Apoptotic DNA Laddering kit (Sigma, St. Louis, MO). The extracts were electrophoresed on 1.2% agarose gel, stained with ethidium bromide and visualized with a UV transiluminator. Alternatively, the *in situ* nuclear DNA fragmentation was determined according to a method based on 3-OH end labeling of DNA breaks with deoxyuridine terminal deoxynucleotidyl transferase. In these studies the cells were incubated for 16 hrs with the test compounds, washed twice with ice-cold PBS containing 4% BSA, fixed with a freshly prepared solution of 4% paraformaldehyde in PBS, resuspended and incubated in a shaking bath for 30 min at room temperature. The fragmented DNA was detected by the "*In Situ* Cell Death Detection" kit (Boehringer Mannheim). Briefly, fluorescein-labeled dUTP was incorporated at the 3'-OH ends of the fragmented DNA by the terminal deoxynucleotidyl transferase reaction (TUNEL). The incorporated nucleotide polymers were detected and quantitated by flow cytometry on a FACS 440 (Becton-Dickinson) flow cytometer with an argon ion laser (Spectra-Physics, Mountain View, CA) tuned to 488 nm (excitation) and 530 nm (emission). The FITC signal was measured with logarithmic amplification (4-decade log scale) and the flow rate was 2000 cells per sec. Consort Vax software (Becton-Dickinson) was used for data acquisition (10,000 events per sample).

Nuclear protein extraction. Cells were harvested and washed twice with ice-cold potassium phosphate buffered saline (PBS) and then resuspended in 120 μl of a first lysis buffer (Hepes-KOH 10 mM, $MgCl_2$ 2 mM, EDTA 0.1 mM, KCl 10 mM, NP40 0.5%, DTT 1 mM, PMSF 0.5 mM, pH 7.9) supplemented with antiproteases (Complete Mini Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). Cells were allowed to swell on ice for 10 min and then vortexed for 8 s. Suspension was then centrifuged at 15000 $\times$ *g* for 30 s and the pellets of nuclei were resuspended in 30 μl of a second lysis buffer (Hepes-KOH 50 mM, $MgCl_2$ 2 mM, EDTA 0.1 mM, NaCl 400 mM, KCl 50 mM, glycerol 10%, DTT 1 mM, PMSF 0.5 mM, pH 7.9) supplemented with antiproteases (Complete Mini Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). Nuclei were allowed to swell on ice for 30 minutes. After centrifugation (15000 $\times$ *g* for 15 minutes), aliquots of supernatant containing the nuclear proteins were stored at -80°C.

Electrophoresis mobility shift assay (EMSA). Electrophoretic Mobility Shift Assays (EMSA) were performed as described.[21] Briefly, 5 μg of nuclear proteins were

incubated for 30 min at room temperature with 0.2 ng of ^{32}P-labeled oligonucleotidic probe, 1 μg of BSA, and 1.3 μg of poly (dI-dC) · poly (dI-dC) (Pharmacia Biotech, The Netherlands) and binding buffer (20 mM HEPES-KOH, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM $MgCl_2$; 1 mM DTT, pH 7.9) in a final volume of 10 μl. DNA-protein complexes were then resolved on a nondenaturing 6% polyacrilamide gel run for 3 h at 300 V in TBE. The gel was then dried and autoradiographied on a Fuji X-ray film (General Electrics, Belgium). The probe (Eurogenetec, Belgium) contains the sequence of NF-κB from the LTR of HIV-1: 5′-GGTTACAAGGGACTTTCCG-CTG-3′. The oligonucleotide probe was labeled by filling in with the Klenow DNA polymerase (Boehringer Manheim, Gemany). Probe (100 ng) was labeled with 3 μCi of (α-^{32}P)-dATP and (α-^{32}P)-dCTP (3000 Ci/mmol; Du Pont de Nemours International, Belgium) and unlabelled dTTP and dGTP (Boehringer Mannheim, Germany), then purified on a Sephadex G-25 (Pharmacia Biotech, The Netherlands) column and stored at $-20°$C until use. Specific radioactivity was always $>10^8$ cpm/μg.

Cell-free caspase-3 activity. 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, 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).

Other assays. Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue,[22] 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. Superoxide dismutase was measured by recording the reduction of NBT[23]; Catalase was measured according to the $TiSO_4$ method[24]; and Glutathione peroxidase activity was assayed by following the oxidation of NADPH as reported by Wendel (1981).[25] The amount of protein content was determined by the method of Lowry *et al.* using BSA as reference.[26]

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

Results

Oxidative stress induced by redox cycling between ascorbate and menadione is the main mechanism involved in their cytotoxicity

Figure 1 shows the survival of TLT cells when ascorbate was associated with menadione. It appeared clearly that cell death was dependent upon menadione concentration: after 24 hours incubation, the combination of ascorbate (2 mM) with menadione at either 2, 4, 8, 10 and 20 μM, induced a LDH leakage of 25, 33, 61, 79 and 86% respectively, as compared to 18% observed in untreated cells (Figure 1A). It should be underlined that this cytotoxic effect was produced when both compounds were added simultaneously since neither ascorbate (2 mM) nor menadione (20 μM) were able to induce cell death when administered separately (Figure 1B).

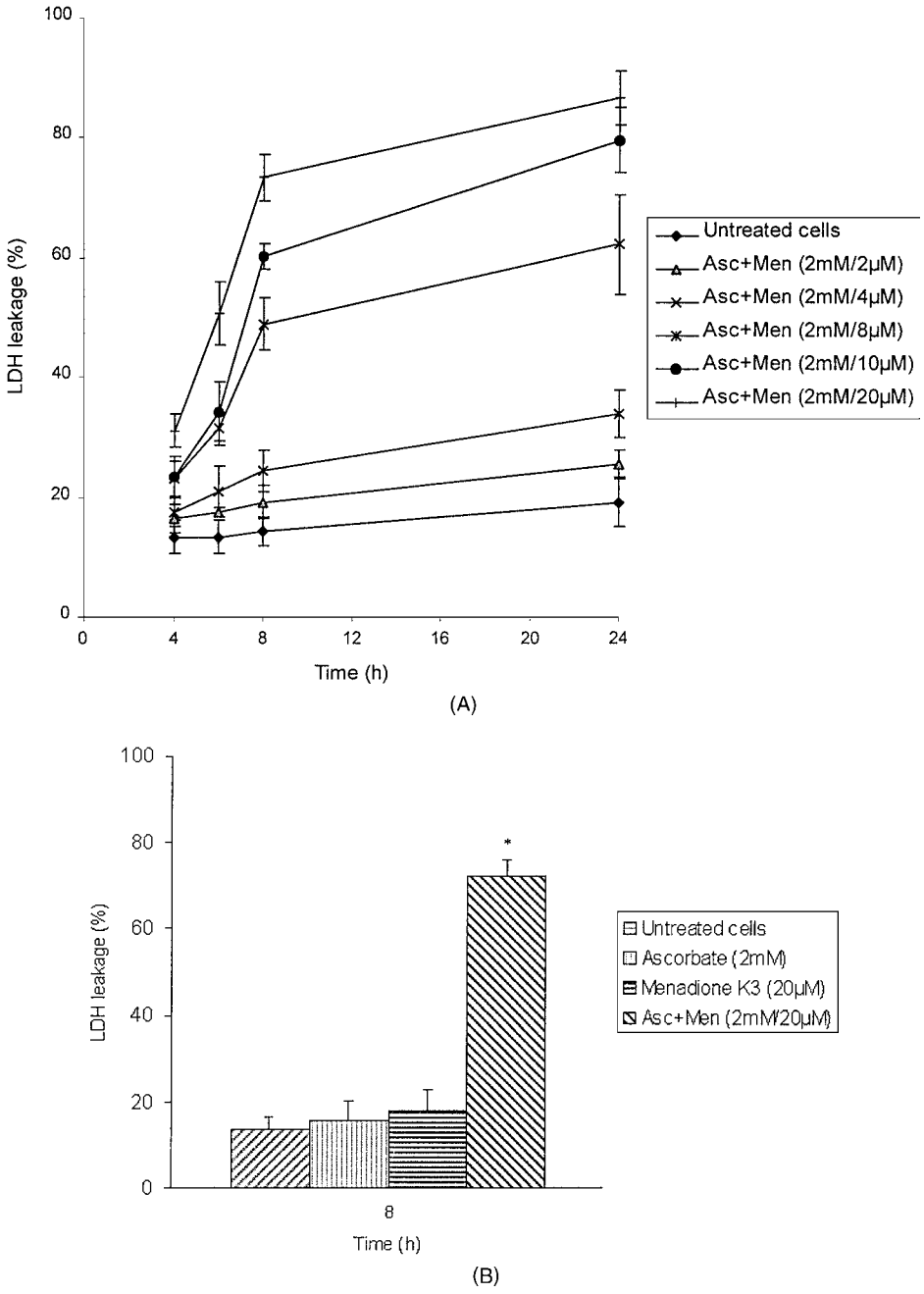
Table 1 shows the basal activites of the three major antioxidant enzymes in TLT cells as compared to values reported in the literature for normal murine hepatocytes.[27] Indeed, the activities of catalase, superoxide dismutase and glutathione peroxidase represented only 5, 1 and 22%, respectively. After 3 hours incubation in the presence of

Table 1. Antioxidant enzymes activities in both normal murine hepatocytes and TLT murine hepatocarcinoma cells. Effect of the association of menadione and ascorbate on TLT antioxidant enzymes

		TLT cells	
Enzymes	**Normal hepatocytes**	**Untreated**	**Treated**
Superoxide dismutase	181 ± 14*	2.15 ± 0.24	1.96 ± 0.26
Catalase	58 ± 3*	3.00 ± 0.50	2.70 ± 0.60
Glutathion peroxidase	24 ± 3*	5.38 ± 0.62	5.84 ± 0.40

TLT cells were incubated for 3 hours in the absence or in the presence of menadione (8 μM) and ascorbate (2 mM). Enzyme activities were measured as described under Materials and Methods. Values are expressed as mUnit/mg protein (glutathione peroxidase and catalase) and as Unit/mg protein (Superoxide dismutase). The results obtained with TLT cells are mean values ± S.E.M. of 3 separated experiments. The addition of menadione and ascorbate alone were without any effect on the three enzyme activities (data not shown).
*Values taken from Sun *et al.*, 1989.

Figure 1. Effect of the association of menadione and ascorbate on cell survival. Cells were incubated for 24 hours in the absence or in the presence of ascorbate (2 mM) combined to different concentrations of menadione ranging from 2 to 20 μM (1A). In Figure 1B, cells were incubated for 8 hours in the absence or in the presence of menadione (20 μM) and ascorbate (2 mM) either alone or in combination. At the indicated times aliquots of cell suspension were taken and LDH leakage was measured as described under Materials and Methods. Results represent the mean values ± SEM from at least 3 experiments. $^*p < 0.05$ as compared with control values (untreated conditions).



the association of menadione/ascorbate (8 μM/2 mM), no modification was observed. Since TLT cells are deficient in the antioxidant defense system, the possibility that the association of menadione and ascorbate shows a selective cytotoxic effect to cancer cells rather than the normal ones has been raised.

The association of menadione and ascorbate kills TLT cells by a caspase-3 independent mechanism

As shown by the TUNEL assay, DNA strand breaks were induced only by the association of menadione and

Table 2. Effect of the association of menadione and ascorbate on DNA fragmentation and caspase-3-like DEVDase activity

Experimental conditions	TUNEL (Mean fluorescence)	DEVDase activity (Unit/mg protein)
Untreated	1.9 ± 0.7	12.6 ± 1.5
Menadione (2 μM)	2.8 ± 0.9	51.8 ± 6.5*
Ascorbate (2 mM)	2.2 ± 0.9	13.9 ± 3.4
Menadione/ascorbate (2 μM/2 mM)	24.5 ± 3.1*	9.5 ± 1.2
Actinomycin D (25 μM)	3.0 ± 0.6	64.2 ± 7.1*
Act + Z-DEVD-FMK (7.5 μM)	n.d.	12.1 ± 1.0$

Cells were incubated for 6 hours (DEVDase), or 16 hours (TUNEL) in the absence or in the presence of menadione and ascorbate either alone or in combination. At the indicated times aliquots of cell suspension were taken and parameters were measured as described under Materials and Methods. The results are expressed as mean values ± SEM from at least 3 experiments. (n.d. = not determined). Cells were preincubated for 1 h in the presence of the caspase-3 inhibitor, before the addition of actinomycin D.

*$p < 0.05$ as compared with control values (untreated conditions).

$$p < 0.05$ as compared with actinomycin D values.

Figure 2. Immunoblotting of pro-caspase-3 and PARP in the presence of menadione and ascorbate. Cells were incubated in the presence of menadione (10 μM) and ascorbate (2 mM) for the different times indicated. Afterwards, cells were washed with PBS and lysed according to procedures described under Materials and Methods. First antibodies were used at a dilution of 1/200 for procaspase-3 and PARP respectively.

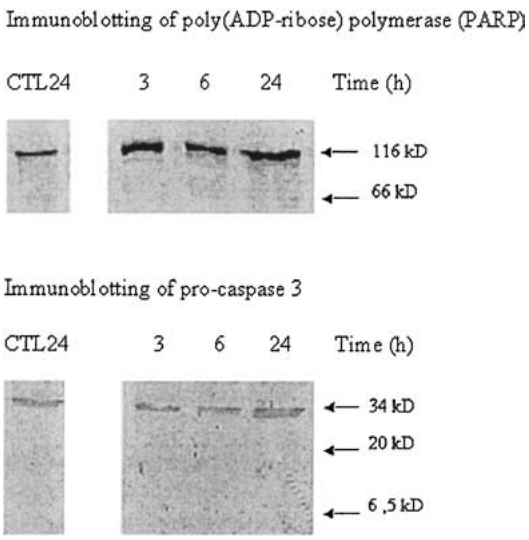
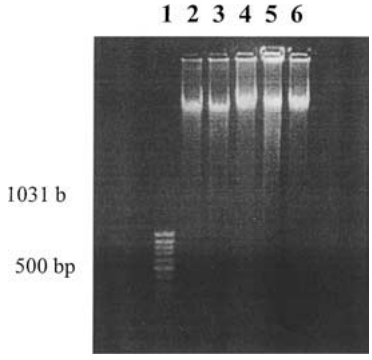


Figure 3. Effect of the association of menadione and ascorbate on DNA fragmentation. Cells were incubated for 16 hours in the absence (lane 2) or in the presence of 2 mM ascorbate (lane 3), 2 μM menadione (lane 4) or a combination of both compounds (lane 5). Actinomycin D was used at 25 μM (lane 6). Genomic DNA was extracted and runned in agarose gel electrophoresis as described under Materials and Methods. Results represented one typical experiment out of three.



ascorbate (Table 2). Such a DNA injury was not the result of cell death since ascorbate (2 mM) was associated with 2 μM menadione, a combination of doses unable to cause cell lysis. Regarding the mechanism, the results in Table 2 also shown that such a DNA fragmentation was unlikely caused by a caspase-3 dependent mechanism. Indeed, after 6 hours incubation no caspase-3 activation was measured in menadione/ascorbate-treated cells but menadione alone and actinomycin D (25 μM), used as positive control, were able to induce 4–5 fold such a caspase-3-like DEVDase activity. Nevertheless, neither menadione alone nor actinomycin D were able to induce cell death, at least until 48 hours incubation (data not shown). It may be possible that such a DEVDase activity involves other caspases. Therefore, in order to exclude a role of caspase-3 in DNA strand breaks by menadione and ascorbate, processing procaspase-3 and the cleavage of one of its specific substrates, namely the poly(ADP-ribose) polymerase (PARP) was investigated. By performing Western-blot procedures it was shown that neither pro-caspase-3 nor PARP were cleaved by the association of menadione and ascorbate (Figure 2). The absence of such a classical apoptotic marker was further confirmed by recording the electrophoretic profile of genomic DNA extracted from cells treated with menadione and ascorbate. The DNA cleavage into oligonucleosomes was indeed not observed but rather a DNA smear pattern (Figure 3). Therefore, apoptosis is unlikely the underlying mechanism explaining such DNA strand breaks.

What is the molecular mechanism involving in cell death caused by the association of menadione and ascorbate?

The previous results show that TLT cells death by the association of menadione and ascorbate is caspase-3 independent but it requires the association of both compounds and, subsequently, the oxidative stress resulting from H_2O_2 generated during their redox cycling. Since

Table 3. Effects of several inhibitors of protein kinases and protein phosphatases on TLT cells death induced by the association of menadione and ascorbate

	LDH leakage (%)	
Treatment	**Untreated cells**	**Treated cells**
H-7 (10 μM)	10.0 ± 1.3	69.6 ± 3.1*
Genistein (50 μM)	10.4 ± 2.0	72.5 ± 4.2*
Okadaic acid (0.1 μM)	15.5 ± 2.2	70.0 ± 3.5*
Sodium vanadate (100 μM)	9.0 ± 0.9	10.2 ± 0.6

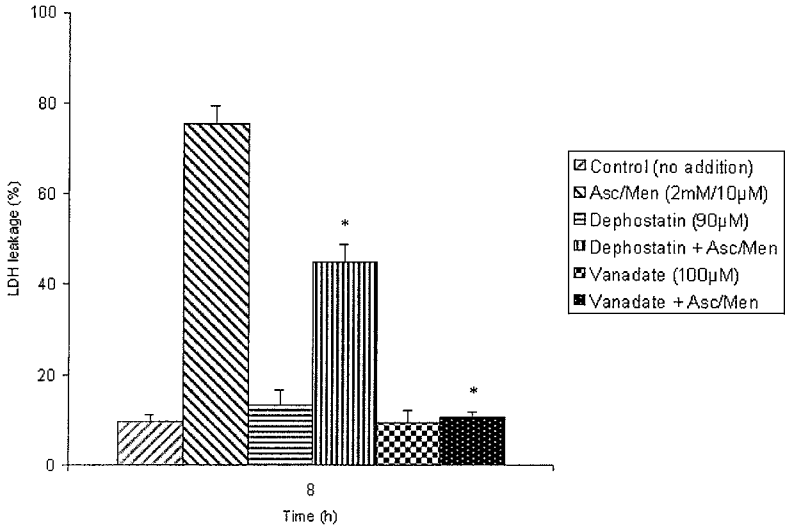
Cells were incubated for 8 hours in the absence or in the presence of the association of menadione (10 μM) and ascorbate (2 mM). Both the kinase and phosphatase inhibitors were tested at the indicated concentrations. LDH leakage was measured as described under Materials and Methods. The results represent the mean values ± SEM from at least 3 experiments.
* $p < 0.05$ compared with untreated cells.

oxidative stress and protein (de)phosphorylation are closely related in controlling cell function and differentiation, including cell death, the putative involvement of protein kinases and phosphatases was investigated (Table 3). Among the different inhibitors, only sodium vanadate, a tyrosine phosphatase inhibitor, entirely suppressed cell lysis induced by the association of menadione and ascorbate. The other inhibitors, namely H-7 (a serine/threonine kinase inhibitor), genistein (tyrosine kinase inhibitor) and okadaic acid (a serine/threonine phosphatase inhibitor) were devoid of a protective effect. The effect of vanadate is unlikely due to redox cycling interference since 3,4-Et-dephostatin, another tyrosine phosphatase inhibitor, was also able to reduce, to a lesser extent, the cytotoxic effect induced by the association of menadione and ascorbate (Figure 4A). In addition, vanadate was not able to modify oxygen uptake that takes place during their redox cycling (data not shown). As shown in Figure 4B, both vanadate and dephostatin strongly enhanced protein phosphorylation in both treated- and untreated-cells indicating that tyrosine phosphatases are, indeed, inhibited. Since Nuclear Factor kappa B (NF-κB), a transcription factor that inhibits apoptosis and favours cancer cell survival, is activated by H_2O_2 and involves protein phosphorylation, its putative role on the menadione/ascorbate-mediated effect was tested. Figure 5 shows an electrophoretic mobility shift assay (EMSA) of different nuclear fractions from cells incubated in the absence or in the presence of menadione and ascorbate either alone or in combination. Hydrogen peroxide added as a bolus (200 μM) was used as positive control and the effect of vanadate on both treated- and untreated cells was also investigated. Unexpectedly, a constitutive activation of NF-κB was observed in untreated cells as well as in cells receiving menadione and ascorbate alone. While H_2O_2 enhance the binding of NF-κB to the probe, the association of menadione and ascorbate produced a strong inhibition. When vanadate was added, a marked activation of NF-κB was observed in all the experimental conditions including that cells incubated with menadione/ascorbate.

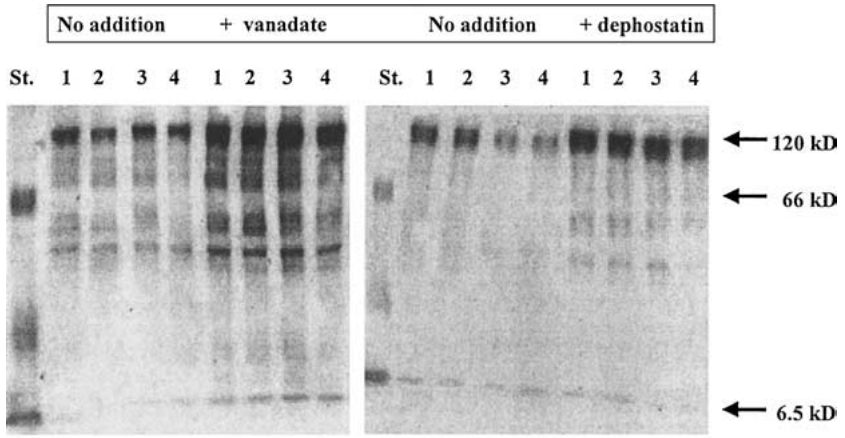
Discussion

A wide variety of cell lines from human origin have been shown to be extremely sensitive to the association of menadione and ascorbate,[4,13,15,16] while non transformed cells such as human gingival fibroblasts, human periodontal ligament fibroblast[15] or rat hepatocytes (unpublished results) were highly resistant. This rather selective effect of the association of menadione and ascorbate to cancer cells is likely due to their extreme sensitivity to oxidative stress because of their low activities of antioxidant enzymes.[27,28] Actually, our results confirm that, in TLT cells, the activities of three major antioxidant enzymes, namely superoxide dismutase, catalase and glutathion peroxidase, are completely depressed as compared to normal murine hepatocytes.[27] Since ascorbate is taken up by cancer cells rather than by normal cells,[29,30] this fact could represent another factor for the selectivity of the treatment towards cancer cells. This selective activity against TLT cells raised the question about the mechanisms conditioning the cell death. It could be argued that the addition of ascorbate acts merely by suppressing the oxidative injury by menadione, as reported by May *et al.* in endothelial cells,[31] but such an hypothesis is very unlikely because cell death induced by the association of menadione and ascorbate was rather increased than suppressed. In agreement with Roginsky *et al.*, who reported that ubiquinone (a benzoquinone derivative) displayed a pronounced catalytic effect on ascorbate oxidation,[32] we suggest that ascorbate reinforces the redox cycling of menadione: in solution, menadione is non-enzymatically reduced by ascorbate to form dehydroascorbate and the semiquinone free radical. Such a semiquinone is rapidly reoxidized to its quinone form by molecular oxygen thus generating reactive oxygen species such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$). Since catalase has a suppressive effect, H_2O_2 is likely the major oxidizing agent involved in the cytotoxicity by the association of menadione and ascorbate.[4,16] The key role of H_2O_2 is also supported by the decrease in the amount of GSH observed in cells treated by menadione/ascorbate, whereas the incubation of such treated cells in the presence of NAC—a well-known GSH precursor[33]—suppressed totally the cytolysis.[16] 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.[34,35] To discriminate which of both pathways (redox cycling or covalent binding) is involved in the cytotoxicity by the association of menadione and ascorbate, DMNQ (2,3-dimethoxy-naphtoquinone), a menadione

Figure 4. Effects of vanadate and 3,4-Et-dephostatin on tyrosine phosphorylation and LDH leakage in control and cells treated with menadione and ascorbate. Cells were incubated for 8 hours in LDH leakage experiments (A) and for 60 min in Western blot experiments (B), in the absence (lanes 1) or in the presence of 2 mM ascorbate (lanes 2), 10 μM menadione (lanes 3), or a combination of both compounds (lanes 4). Sodium orthovanadate and 3,4-Et-dephostatin were added at 100 μM and 90 μM, respectively. At the indicated times aliquots of cell suspension were taken and cellular survival and levels of tyrosine phosphorylation (by immunoblotting of phosphotyrosines) were evaluated as described under Materials and Methods. Results of LDH leakage represents mean values ± SEM from at least 3 experiments, while the fluorogram is a typical of out of three experiments. $^{*}p < 0.05$ as compared with control values (untreated conditions).



(A)

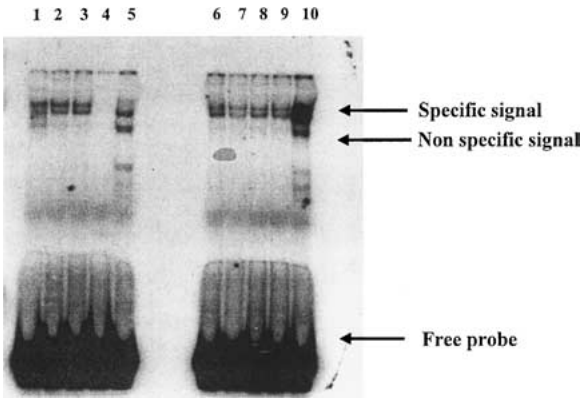


(B)

analog without arylation sites, was used. The association of ascorbate with DMNQ instead of menadione, produced the same profile of cytotoxicity, underlining the key role of the redox cycling pathway.[16]

Since cancer cells pass across regulatory check-points and survive to different forms of programmed cell death, the activation of apoptosis has become a major therapeutic cancer cells target. Apoptosis is characterized by DNA fragmentation thus yielding a characteristic "laddering" on agarose gel electrophoresis.[36] It is initiated by a variety of stimuli, including exposure to a number of cytokines, removal of growth factors and oxidant injury.[37–40] Up to now, the final common element in the initiation for all of the apoptotic pathways is the activation of a family of a cysteine-aspartate specific proteases, the caspases,[38,39] and in particular, caspase-3. A number of studies in various cell lines has suggested that menadione toxicity is mediated through apoptosis,[41–43] whereas other authors reported that both necrosis and apoptosis are involved in menadione cytotoxicity.[44] Concerning ascorbate, Sakagami *et al.* reported an induction of apoptotic cell death in group I cells (such as HL-60, ML-1, U-937, THP-1), but not in group II cells (such as normal human leukocytes, and cell lines like K562, Molt-4, EOL-1, Colo

Figure 5. Activation of NF-κB in cells treated by the association of menadione and ascorbate. Cells were incubated for 16 hours in the absence (lanes 1 and 6) or in the presence of 2 mM ascorbate (lanes 2 and 7), 2 μM menadione (lanes 3 and 8), or a combination of both compounds (lanes 4 and 9). Hydrogen peroxide added as a bolus at 200 μM was used as positive test (lanes 5 and 10). The activation of NF-κB was assessed in the absence of vanadate (lanes 1–5) or in the presence of 100 μM of vanadate (lanes 6–10). At the indicated times aliquots of cell suspension were taken and samples were processed as described under Materials and Methods. Experiments were performed in duplicate.



201, etc), most probably by difference in chromatin structure between both groups of cells.[45] In order to identify the type of cell death caused by the association of menadione and ascorbate, several markers were investigated. Our results support the hypothesis made by Gilloteaux *et al.*[17] They showed that cell death induced by this association is mainly occurring by autoschizis and rarely by apoptosis. What experimental data do we have to support this assessment?

(1) The activation of caspase-3—a hallmark of apoptosis[38,39]—was observed in cells treated with menadione alone but did not occur in the presence of the association of menadione and ascorbate. Conversely, this association was able to cause cell death whereas menadione alone did not.
(2) Actinomycin D did not induce cell death in a period of 48 hours but it activated caspase-3 after 6 hours incubation. Such an activation was completely suppressed by the caspase inhibitor Z-DEVD-FMK.
(3) Oxidative stress induced by the association of menadione and ascorbate generates H_2O_2 that can oxidize the critical cystein residue, within the QACRG motif in the caspase catalytic site,[46–47] rendering the enzyme inactive.
(4) The severe ATP depletion caused by the association of menadione and ascorbate[16] may prevent the formation of the apoptosome complex and block the recruitment and processing of caspase-9.[48]
(5) DNA strand breaks are occurring in cells treated with the association of menadione and ascorbate as shown by the TUNEL assay, but such a DNA fragmentation did not show a DNA laddering, another hallmark of apoptosis.[36]
(6) As shown previously,[16] a complex pattern of cell death is observed in both treated- and untreated TLT cells. On the basis of morphological evaluation (Giemsa staining) and flow cytometry analysis (Annexin-V and propidium iodide labeling) it is suggested that TLT cells treated by the association of menadione and ascorbate die mainly by autoschizis.

What are the signal transduction pathways involved in autoschizis? To answer that question, we focused our study on protein kinases because it is generally accepted that they play a major role in controlling cell function and differentiation, including cell death.[49,50] Our results show that vanadate, a well-known inhibitor of protein tyrosine phosphatases, completely suppresses the cytotoxicity induced by the association of menadione and ascorbate, while other inhibitors of protein kinases (H7 and genistein for tyrosine and serine/threonine kinases respectively) and protein phosphatases (okadaic acid for serine/threonine phosphatases) did not modify at all such a cytotoxicity. The complete suppression by vanadate of the cytotoxicity induced by the association of menadione and ascorbate might be explained by three different ways: the first one involves a potential interfering reaction by vanadate in redox cycling, but is very unlikely due to the vanadate redox potential. The second is related to the formation of peroxovanadate, a possible reaction of vanadate with H_2O_2.[51] Such a reaction might consume most (or all) of H_2O_2 thus avoiding its interaction with the intracellular putative targets. The third possibility is that vanadate (or peroxovanadate) by inhibiting tyrosine phosphatases, modifies the phosphorylation state of some critical proteins. The results obtained with another tyrosine phosphatase inhibitor, namely Et-3,4-dephostatin,[52] confirms that the protective effect by vanadate may be attributed to an inhibition of tyrosine phosphatases. Nevertheless, the specific proteins that are involved in this process have not been yet identified.

Finally, among different transcription factors, we investigated a putative role of NF-κB because it is involved in cell death and proliferation. It has been proposed that NF-κB inhibits apoptosis and favors cancer cell survival.[53,54] For instance, the activation of NF-κB is protecting HepG2 cells against cytotoxicity by menadione.[55] Under physiological conditions, NF-κB is present in its inactive state in the cytoplasm as a complex with I-κB. This latter is degraded by the proteasome after phosphorylation and ubiquitination reactions. This allows the release of NF-κB and its translocation to the nucleus, where it binds to the promoter region of DNA and activates genes that mediate carcinogenesis and metastasis.[56] Three lines of evidence are connecting the cytotoxicity induced by

the association of menadione and ascorbate and NF-κB activation:

- H_2O_2, the main oxidizing agent involved in cancer cell death by the association of menadione and ascorbate, has been shown to induce the activation of NF-κB;[57]
- N-acetylcysteine, a precursor of GSH, has been shown to inhibit both the activation of NF-κB[57] and the cytotoxicity by menadione/ascorbate.[16]
- Vanadate increases protein phosphorylation while the activation of NF-κB needs that I-κB should be phosphorylated.

Our results show that the association of menadione and ascorbate is inhibiting rather than activating NF-κB, that is the opposite of the expected effect. Interestingly, the results obtained with other compounds showing a similar cytotoxicity profile like sanguinarine that also inhibited NF-κB activation,[58] led us to conclude that in some cancer cell types, the activation of NF-κB is constitutive, and by this way cancer cells increase their survival capacity. The inhibition of NF-κB activation represents then one of the mechanisms explaining the cell death induced by the association of menadione and ascorbate. A second question is related to the role played by H_2O_2, since its transient addition is producing a totally different effect as compared to its permanent formation. A direct addition of H_2O_2 as a bolus (200 μM) results in a strong activation of NF-κB while its generation during the redox cycling inhibited the constitutive NF-κB activation. In the presence of vanadate, the inhibitory effect of the association of menadione and ascorbate was lost and NF-κB was reactivated. We do not have a full explanation for such a difference but it is clear that the exposure-time and H_2O_2 concentration play an important role. Moreover, although reactive oxygen species have been proposed to act as second messengers to activate NF-κB,[57] several cell types have been shown to be insensitive to H_2O_2, suggesting that activation of NF-κB by H_2O_2 may be the exception rather than the rule.[59]

Conclusion

Our results indicate that cell death resulted from a synergistic effect from menadione/ascorbate redox cycling, that it was caspase-3 independent, that it involved tyrosine phosphorylation and that it did not require NF-κB activation. Taking together with previous results[16,19] such as phosphatidylserine asymetry changes (flow cytometry) and the morphological features of dying cells (Giemsa staining), and the initial remark made by Gilloteaux *et al.* about membrane damage with a progressive loss of organelles-free cytoplasm,[17] we suggest that autoschizis is the predominant form of cell death induced by the association of menadione and ascorbate.

Acknowledgements

The authors thanks Isabelle Blave for her excellent technical assistance.

References

1. Wu FY, Sun TP. Vitamin K_3 induces cell cycle arrest and cell death by inhibiting Cdc25 phosphatase. *Eur J Cancer* 1999; **35**: 1388–1393.
2. Hollensworth SB, Shen C, Sim JE, Spitz DR, Wilson GL, Ledoux SP. Glial cell type-specific responses to menadione-induced oxidative stress. *Free Rad Biol Med* 2000; **28**: 1161–1174.
3. Warren MC, Bump EA, Medeiros D, Braunhut SJ. Apoptosis-inducing activity of vitamin C and vitamin K. *Free Rad Biol Med* 2000; **29**: 537–547.
4. Noto V, Taper HS, Jiang YH, Janssens J, Bonte J, De Loecker W. Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K3) treatment on human tumor cell growth *in vitro*. I. Synergism of combined vitamin C and K3 action. *Cancer* 1989; **63**: 901–906.
5. Taper HS, Brucher JM, Fort L. Alkaline and acid nucleases activity in the tumors of the human central nervous system (histochemical study). *Cancer* 1971; **28**: 482–490.
6. Taper HS. Reversibility of acid and alkaline deoxyribonuclease deficiency in malignant tumor cells. *J Histochem Cytochem* 1981; **29**: 1053–1060.
7. Taper HS, Deckers CO, Deckers-Passau LO. Increase in nuclease activity as a possible means for detecting tumor cell sensitivity to anticancer agents. *Cancer* 1981; **47**: 523–529.
8. De Loecker W, Janssens J, Bonte J, Taper HS. Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphtoquinone (vitamin K3) treatment on human tumor cell growth *in vitro*. II. Synergism with combined chemotherapy action. *Anticancer Res* 1993; **13**: 103–106.
9. Taper HS, De Gerlache J, Lans M, Roberfroid M. Non-toxic potentiation of cancer chemotherapy by combined C and K_3 vitamin pre-treatment. *Int J Cancer* 1992; **40**: 575–579.
10. Taper HS, Roberfroid M. Non-toxic sensitization of cancer chemotherapy by combined vitamin C and K_3 pretreatment in a mouse tumor resistant to oncovin. *Anticancer Res* 1992; **12**: 1651–1654.
11. Taper HS, Keyeux A, Roberfroid M. Potentiation of radiotherapy by non-toxic pretreatment with combined vitamins C and K_3 in mice bearing solid transplantable tumor. *Anticancer Res* 1996; **96**: 499–503.
12. Gilloteaux J, Jamison JM, Venugopal M, Giammar D, Summers JL. Scanning electron microscopy and transmission electron microscopy aspects of synergistic antitumor activity of vitamin C—Vitamin K3 combinations against human prostatic carcinoma cells. *Scanning Microscopy* 1995; **9**: 159–173.
13. Venugopal M, Jamison JM, Gilloteaux J, *et al.* Synergistic antitumor activity of vitamins C and K3 on human urologic tumor cell lines. *Life Sci* 1996; **59**: 1389–1400.
14. Sakagami H, Satoh K, Hakeda Y, Kumegawa M. Apoptosis-inducing activity of vitamin C and vitamin K. *Cell Mol Biol* 2000; **46**: 129–143.

15. Zhang W, Negoro T, Satoh K, *et al.* Synergistic cytotoxic action of vitamin C and vitamin K3. *Anticancer Res* 2001; **21**: 3439–3444.
16. Buc Calderon P, Cadrobbi J, Marques C, *et al.* Potential therapeutic application of the association of vitamins C and K_3 in cancer treatment. *Cur Med Chem* 2002; **9**: 2271–2285.
17. Gilloteaux J, Jamison JM, Arnold D, *et al.* Cancer cell necrosis by autoschizis: Synergism of antitumor activity of vitamin C: Vitamin K3 on human bladder carcinoma T24 cells. *Scanning* 1998; **20**: 564–575.
18. Jamison JM, Gilloteaux J, Taper HS, Buc Calderon P, Summers JL. Autoschizis: A novel cell death. *Biochem Pharmacol* 2002; **63**: 1773–1783.
19. Verrax J, Cadrobbi J, Delvaux M, *et al.* The association of vitamins C and K3 kills cancer cells mainly by autoschizis, a novel form of cell death. Basis for their potential use as coadjuvants in anticancer therapy. *Eur J Med Chem* 2003; **38**: 451–457.
20. Naora H, Nishida T, Shindo Y, Adachi M, Naora H. Constitutively enhanced nbl expression is associated with the induction of internucleosomal DNA cleavage by actinomycin *D. Biochem Biophys Res Commun* 1996; **224**: 258–264.
21. Legrand-Poels S, Bours V, Piret B, *et al.* Transcription factor NF-κB is activated by photosensitization generating oxidative DNA damages. *J Biol Chem* 1995; **270**: 6925–6934.
22. Wrobleski F, Ladue J. Lactic deshydrogenase activity in blood. *Proc Soc Exp Biol Med* 1955; **90**: 210–213.
23. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969; **244**: 6049–6055.
24. Baudhuin P, Beaufay H, Rahman-Li Y, *et al.* Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat liver tissue. *Biochem J* 1964; **92**: 179–184.
25. Wendel A. Glutathione peroxidase. *Methods Enzymol* 1981; **77**: 325–333.
26. Lowry O, Rosebrough N, Farr L, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **183**: 265–275.
27. Sun Y, Oberley LW, Elwell JH, Sierra-Rivera E. Antioxidant enzyme activities in normal and transformed mouse liver cells. *Int J Cancer* 1989; **44**: 1028–1033.
28. Oberley TD, Oberley LW. Antioxidant enzyme levels in cancer. *Histol Histopathol* 1997; **12**: 525–535.
29. Bodansky O, Wrobleski D, Makardt B. Concentration of ascorbic acid in plasma and white cells of patients with cancer and noncancerous chronic diseases. *Cancer Res* 1951; **11**: 238–242.
30. Lloyd V, Davis PS, Emery H, Lauder H. Platelet ascorbic acid levels in normal subjects and in diseases. *J Clin Pathol* 1972; **25**: 478–483.
31. May JM, Qu ZC, Li X. Ascorbic acid blunts oxidant stress due to menadione in endothelial cells. *Arch Biochem Biophys* 2003; **411**: 136–144.
32. Roginsky VA, Bruchelt G, Bartuli O. Ubiquinone-0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone) as effective catalyzer of ascorbate and epinephrine oxidation and damager of neuroblastoma cells. *Biochem Pharmacol* 1998; **55**: 85–91.
33. Thor H, Moldeus P, Orrenius S. Metabolic activation and hepatotoxicity. Effect of cysteine, N-acetylcysteine, and methionine on glutathione biosynthesis and bromobenzene toxicity in isolated rat hepatocytes. *Arch Biochem Biophys* 1979; **192**: 405–413.
34. Gant TW, Rao DN, Mason RP, Cohen GM. Redox cycling and sulphydryl arylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. *Chem Biol Interactions* 1988; **65**: 157–173.
35. Grant TW, Doherty MD, Odowole D, Sales KD, Cohen GM. Semiquinone anion radicals formed by the reaction of quinones with glutathione or amino acids. *FEBS Lett* 1986; **201**: 296–300.
36. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980; **284**: 555–556.
37. Uren AG, Vaux DL. Molecular and clinical aspects of apoptosis. *Pharmacol Ther* 1996; **72**: 37–50.
38. Cohen GM. Caspases: The executioners of apoptosis. *Biochem J* 1997; **325**: 1–16.
39. Nicholson DW, Thornberry NA. Caspases: Killer proteases. *Trends Biochem Sci* 1997; **22**: 299–306.
40. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309–1312.
41. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; **75**: 241–251.
42. Lindenboim L, Haviv R, Stein R. Inhibition of drug-induced apoptosis by survival factors in PC12 cells. *J Neurochem* 1995; **64**: 1054–1063.
43. Castedo M, Hirsch T, Susin SA, *et al.* Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J Immunol* 1996; **157**: 512–521.
44. Sata N, Klonowski-Stumpe H, Han B, Haussinger D, Niederau C. Menadione induces both necrosis and apoptosis in rat pancreatic acinar AR4-2J cells. *Free Rad Biol Med* 1997; **23**: 844–850.
45. Sakagami H, Satoh K. Modulating factors of radical intensity and cytotoxic activity of ascorbate. *Anticancer Res* 1997; **17**: 3513–3520.
46. Hampton MB, Orrenius S. Dual regulation of caspase activity by hydrogen peroxide: Implications for apoptosis. *FEBS Lett* 1997; **414**: 552–556.
47. Li J, Billiar TR, Talanian RV, Kim YM. Nitric Oxide Reversibly Inhibits Seven Members of the Caspase Family via S-Nitrosylation. *Biochem Biophys Res Commun* 1997; **240**: 419–424.
48. Bratton SB, Cohen GM. Apoptotic death sensor: An organelle's alter ego? *Trends Pharmacol Sci* 2001; **22**: 306–315.
49. Gauld SB, Blair D, Moss CA, Reid SD, Harnett MM. Differential roles for extracellularly regulated kinase-mitogen-activated protein kinase in B cell antigen receptor-induced apoptosis and CD40-mediated rescue of WEHI-231 immature B cell. *J Immunol* 2002; **168**: 3855–3864.
50. Pimentel B, Rodriguez-Bolardo L, Hernandez C, Carrera AC. A role for phosphoinositide 3-kinase in the control of cell division and survival during retinal development. *Develop Biol* 2002; **247**: 295–306.
51. Huyer G, Liu S, Kelly J, *et al.* Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. *J Biol Chem* 1997; **272**: 843–851.
52. Watanabe T, Suzuki T, Umezawa Y, *et al.* Structure-activity relationship and rational design of 3,4-dephostatin derivatives as protein tyrosine phosphatase inhibitors. *Tetrahedron* 2000; **56**: 741–752.
53. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: Potentiation by inhibition of NF-kappaB. *Science* 1996; **274**: 784–787.

54. Aggarwal BB. Apoptosis and nuclear factor-kappa B: A tale of association and dissociation. *Biochem Pharmacol* 2000; **60**: 1033–1039.
55. Chen Q, Cederbaum AI. Menadione cytotoxicity to Hep G2 cells and protection by activation of nuclear factor-kappaB. *Mol Pharmacol* 1997; **52**: 648–657.
56. Bharti AC, Aggarwal BB. Nuclear factor-kappa B and cancer: Its role in prevention and therapy. *Biochem Pharmacol* 2002; **64**: 883–888.
57. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 1991; **10**: 2247–2258.
58. Chaturvedi MM, Kumar A, Darnay BG, Chainy BG, Aggarwal S, Aggarwal BB. Sanguinarine (pseudochelerythrine) is a potent inhibitor of NF-kappaB activation, IkappaBalpha phosphorylation, and degradation. *J Biol Chem* 1997; **272**: 30129–30134.
59. Bowie A, O'Neill LA. Oxidative stress and nuclear factor-kappaB activation: A reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 2000; **59**: 13–23.