Part 2: Influence of 2-Euryfuryl-1,4-naphthoquinone and Its *peri*-Hydroxy Derivatives on Both Cell Death and Metabolism of TLT Cells, a Murine Hepatoma Cell Line. Modulation of Cytotoxicity by Vitamin C

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2-Euryfuryl-1,4-naphthoquinone C_1 and its 5- and 5,8-hydroxy derivatives C_2 and C_3, were tested for their cytotoxicity towards transplantable liver tumor (TLT) cells (a murine hepatoma cell line) in the absence and in the presence of vitamin C. Cell death, caspase-3 activity and two metabolic end-points, namely the intracellular content of ATP and glutathione (GSH), were employed to evaluate their cytotoxicity. In a range of concentration from 0 to 10 μg/ml C_1 and C_3 were non toxic against TLT cells, while compound C_2 killed about 50% of cells by necrosis. Interestingly, the presence of vitamin C did not enhance the cytolysis of C_2, but its addition exacerbated the effects of the three compounds on both ATP and GSH contents, the two metabolic end points selected in our study. Our assumption is that the electron donor effect of the *peri*-hydroxyl substituents on euryfurylnaphthoquinones and the hydrogen bond between the *peri*-hydroxy and quinone carbonyl groups influence the electron-acceptor capability of the quinone nucleus and thus modifies the electron transfer from ascorbate to the electroactive quinone nucleus. The combination of euryfurylnaphthoquinones with vitamin C may be of potential clinical interest, because cancer cells accumulate vitamin C, they are sensitive to an oxidant insult and they depend on glycolysis (ATP formation) for their survival.

Key words cancer; cell death; naphthoquinone; oxidative stress; vitamin C

Cancer, second cause of mortality in the world (about 1700000 deaths in Europe in 2006)[1] is a leading life-threatening pathology characterized by a deregulation of the cell cycle which results in a progressive loss of the cellular differentiation and a non-controlled cellular growth. In spite of the enormous diversity of human cancers, cancer cells have acquired the same set of functional capabilities: self sufficiency in growth signals, insensitivity to growth signals, sustained angiogenesis, limitless replication potential, tissue invasion and metastasis, and evasion of apoptosis.[2] Moreover, cancer cells are presenting an almost universal glycolytic phenotype,[3] a deficiency in antioxidant enzymes like catalase and superoxide dismutase,[4,5] and a trend to accumulate vitamin C.[6]

Interestingly, two vitamins, namely ascorbate (vitamin C) and menadione (vitamin K_3) were reported to potentiate both chemo- and radiotherapy in tumor-bearing mice.[7,8] It should be noted that the action of these compounds is not related to their action as vitamins, but rather involves a cytolytic process that takes advantage of tumor metabolism. Indeed, we have shown that the combination of ascorbate and menadione generates oxidative stress, leads to the inhibition of glycolysis and induces a necrotic cell death.[9,10] This mechanism could be of particular interest in cancer therapy for several reasons. First, since many tumors exhibit defects in the apoptotic signaling pathways, there is a need for additional mechanisms to induce cell death. Second, as mentioned previously, cancer cells are expected to be more sensitive towards an oxidant stress than healthy cells because they are often deficient in antioxidant defenses. Third, due to the high energetic dependence of cancer cells on glycolysis, the impairment of such an essential pathway will be critical for their survival.

We took advantage of these features to develop an original approach that consists in the exposure of cancer cells to an oxidant insult. Indeed, a redox cycle is initiated by electron transfer from ascorbate to menadione leading to the formation of a semiquinone free radical. The rapid reoxidation of the semiquinone to its quinone form by molecular oxygen leads to generation of reactive oxygen species (ROS). Among them, we have recently reported that: first, the main ROS formed is H_2O_2, and second, it may be formed by redox cycling between ascorbate and other quinones depending on their half-redox potentials and the stabilities of the semiquinone species.[11]

As part of our current research program on sesquiterpene quinones we have recently reported a study with 2-euryfuryl- and 2-euryfuryl-3-nitro-1,4-benzoquinone on their cytotoxicity towards transplantable liver tumor (TLT) cells in the absence and in the presence of vitamin C.[12] Our findings revealed that inactivated 2-euryfuryl-3-nitro-1,4-benzoquinone could undergo an activation process by a redox mechanism causing cell death on TLT cells, whereas 2-euryfurylbenzoquinone, which would be less capable of bioreductive activation, seems to induce apoptosis (as shown by the increased activity of caspase-3), irrespective of the presence or the absence of vitamin C.[12]

In continuation of these studies we focused our attention on 2-euryfuryl-1,4-naphthoquinone $\mathbf{C}_1$ and its 5- and 5,8-hydroxyderivatives $\mathbf{C}_2$ and $\mathbf{C}_3$. Our interest in these particular compounds is related to the influence of both the electronic and hydrogen bond effects of hydroxy groups located at the *peri* position of $\mathbf{C}_1$ on the oxidative stress-mediated effects.

Here we wish to report our results with 2-euryfury-1,4-

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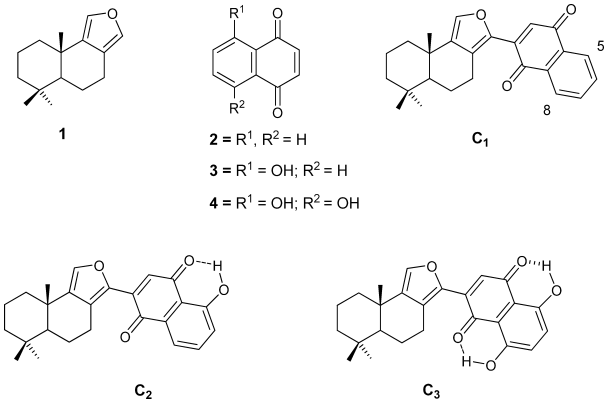

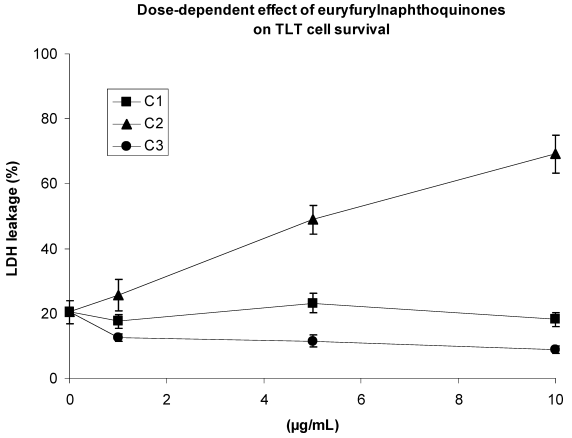

Fig. 1. Effect of Di-, Mono- and Non-hydroxylated Euryfurylnaphthoquinones on Cell Survival

TLT cells were incubated for 24 h at 37 °C in the presence of $\mathbf{C}_1$ (squares), $\mathbf{C}_2$ (triangles), and $\mathbf{C}_3$ (circles) at different concentrations varying from 0 to 10 μg/ml. Aliquots of cell suspension were taken and the activity of LDH was measured as indicated in Experimental. The results are expressed as % of LDH leakage and represent the mean values±S.E.M. of three different experiments. The effects of $\mathbf{C}_3$ were significantly different from the two other experimental groups ($p<0.05$).

naphthoquinone $\mathbf{C}_1$ and its 5- and 5,8-hydroxyderivatives $\mathbf{C}_2$ and $\mathbf{C}_3$ on both cell death and some metabolic markers in TLT cells, a murine hepatoma cell line that is strongly deficient in antioxidant enzymes as compared to normal murine hepatocytes.[5] In addition, we investigate a potential modulatory effect by vitamin C on cell death induced by euryfurylnaphthoquinones. Cell survival was assessed by measuring the release of the cytoplasmic lactate dehydrogenase (LDH) into the incubation medium. Two metabolic end-points, namely the intracellular contents of ATP and glutathione (GSH), were employed to evaluate the cytotoxicity of 2-euryfuryl-1,4-naphthoquinone $\mathbf{C}_1$ and its 5- and 5,8-hydroxy derivatives $\mathbf{C}_2$ and $\mathbf{C}_3$. In addition, the putative effects of these quinones on caspase-3 activity were assessed by measuring the proteolytic cleavage of a synthetic fluorchrome peptide (DEVD-AFC).

Chemistry Compounds $\mathbf{C}_1$ (2-euryfuryl-1,4-naphthoquinone), $\mathbf{C}_2$ (2-euryfuryl-8-hydroxy-1,4-naphthoquinone) and $\mathbf{C}_3$ (2-euryfuryl-5,8-dihydroxy-1,4-naphthoquinone) were prepared through oxidative coupling reactions of (+)-euryfuran **1**, an antitumoral drimane, with 1,4-naphthoquinone **2**, 5-hydroxy-1,4-naphthoquinone **3** (juglone) and 5,8-dihydroxy-1,4-naphthoquinone **4** (naphtazarin), according to a previously reported procedure.[13,14]

In the present study, quinones $\mathbf{C}_1$, $\mathbf{C}_2$ and $\mathbf{C}_3$ were selected as representative compounds for two main reasons. First, the introduction of electron-donor hydroxyl groups into the *peri*-positions (C-5 and C-8) of 2-euryfuryl-1,4-naphthoquinone $\mathbf{C}_1$, as in compounds $\mathbf{C}_2$ and $\mathbf{C}_3$, should decrease the capacity of the electroactive group to undergo a reductive process. Second, the hydrogen bond between the *peri*-hydroxy and quinone carbonyl groups, should increase the capacity of the electroactive group to undergo a reductive process. Therefore, the capability of the electroactive quinone nucleus of compounds $\mathbf{C}_2$ and $\mathbf{C}_3$ will finally depend of the magnitude of these two opposite processes. Based on these assumptions, we anticipate that the presence of *peri*-hydroxy groups in $\mathbf{C}_2$ and $\mathbf{C}_3$ should affect the electron transfer from ascorbate (vitamin C) to the electroactive quinone nucleus compared with the same process on euryfurylnaphthoquinone $\mathbf{C}_1$.

Biology Murine hepatocarcinoma cells, namely transplantable liver tumors (TLT), 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—2×10^5 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.

TLT cells were incubated for 24 h at 37 °C in the absence or in the presence of $\mathbf{C}_1$, $\mathbf{C}_2$ and $\mathbf{C}_3$ at different concentrations (1, 5, 10 μg/ml). In addition, cells (both untreated and treated), were incubated in the presence of 2 mM vitamin C. Sodium ascorbate (vitamin C) and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were ACS reagent grade.

Results

Biology Figure 1 shows the effect of three euryfurylnaphthoquinones derivatives $\mathbf{C}_1$, $\mathbf{C}_2$ and $\mathbf{C}_3$ (non substituted, mono-hydroxylated and di-hydroxylated respectively) on TLT cells survival as a function of their concentration. In the overall range of concentration (from 0 to 10 μg/ml), neither $\mathbf{C}_1$ nor $\mathbf{C}_3$ were able to modify the survival of TLT cells. Indeed, LDH leakage values remain fairly stable at around 10—20%. However, the incubation of TLT cells in the presence of $\mathbf{C}_2$ results in an increasing loss of cell survival reaching values of 50% and 70% of LDH leakage, at 5 and 10 μg/ml respectively.

Figure 2a shows no difference in cell death induced by $\mathbf{C}_2$ (5 μg/ml) alone or in the presence of 2 mM vitamin C. Indeed, cell death was around 50% under both conditions. Furthermore, the addition of 2 mM vitamin C did not modify the survival of TLT cells by either $\mathbf{C}_1$ or $\mathbf{C}_3$, when tested at 5 μg/ml.

Figure 2b shows the effect of the three euryfurylnaphthoquinones $\mathbf{C}_1$, $\mathbf{C}_2$ and $\mathbf{C}_3$ (at 1 μg/ml), both in the absence and in the presence of vitamin C (2 mM), on ATP and GSH intracellular content. It should be underlined that at this concentration, none of the euryfurylnaphthoquinones affect the survival of TLT cells.

Regarding the ATP content, the effect of the compounds, in the absence of vitamin C, was completely different: as compared to control values both $\mathbf{C}_1$ and $\mathbf{C}_2$ had no effect but, unexpectedly, $\mathbf{C}_3$ increased the ATP content by 31%. In contrast to the effects observed on cell survival, the addition of

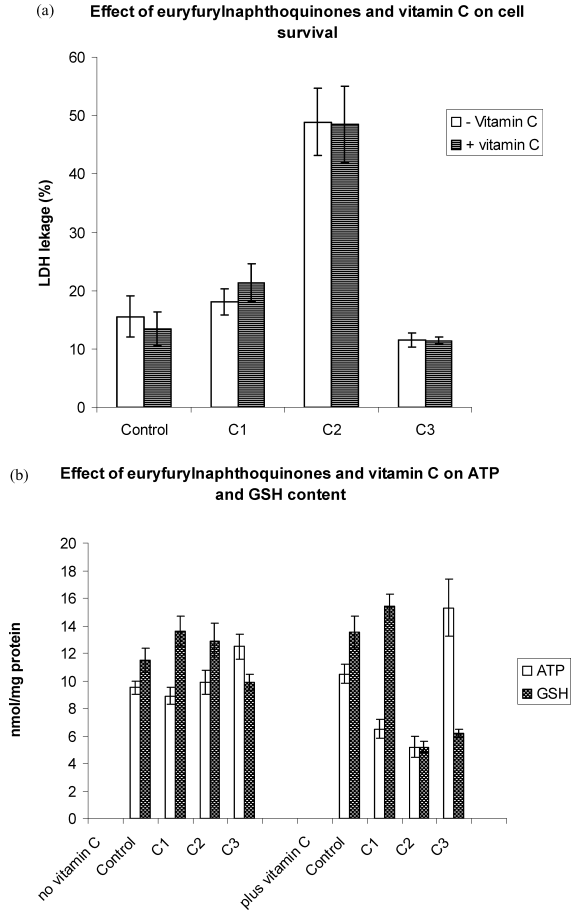

Fig. 2. Influence of Vitamin C on Both Cell Death and Metabolic Effects Induced by Di-, Mono- and Non-hydroxylated Euryfurylnaphthoquinones

(a) TLT cells were incubated for 24 h at 37 °C with 5 μg/ml of each tested compound in the absence (open bars) and in the presence (solid bars) of 2 mM vitamin C. Aliquots of cell suspension were taken and the activity of LDH was measured as indicated in Experimental. The results are expressed as % of LDH leakage and represent the mean values±S.E.M. of three different experiments. The effects of $\mathbf{C}_2$ were significantly different from the other experimental groups ($p<0.05$). (b) TLT cells were incubated for 24 h at 37 °C with 1 μg/ml of each selected compounds in the absence and in the presence of 2 mM vitamin C (VC). Aliquots of cell suspension were taken and the content of ATP (open bars) and GSH (closed bars) was measured as indicated in Experimental. The results are expressed as nmol/mg of proteins and represent the mean values±S.E.M. of three different experiments. In the absence of vitamin C, the effect of $\mathbf{C}_3$ was significantly different from the three other experimental groups ($p<0.05$). In the presence of vitamin C, regarding ATP content, all euryfurylnaphthoquinones were different ($p<0.05$) to the control group. With regard to GSH levels, both $\mathbf{C}_2$ and $\mathbf{C}_3$ were different ($p<0.05$) to control and $\mathbf{C}_1$.

vitamin C (2 mM) exacerbated the effects of the three euryfurylnaphthoquinones on this metabolic end-point. For instance, as compared to untreated TLT cells, $\mathbf{C}_1$ and $\mathbf{C}_2$ decreased the content of ATP by 38% and 50%, respectively, while $\mathbf{C}_3$ increased it by 46%.

The effects of the three euryfurylnaphthoquinones on the second metabolic marker, namely, the levels of GSH, also show a different profile. Indeed, in the absence of vitamin C, the 3 quinones were unable to modify the intracellular levels of GSH. In the presence of vitamin C, however, both $\mathbf{C}_2$ and $\mathbf{C}_3$ decrease the intracellular levels of GSH by about 61% and 54%, respectively. $\mathbf{C}_1$ has no effect on levels of GSH.

Finally, Table 1 shows the effects of euryfurylnaphthoquinones (at 5 μg/ml) in the absence or in the presence of vitamin C on caspase-3 activation. Whatever the condition analysed, no activation of caspase-3 was detected. The incubation of TLT cells with Sanguinarine (10 μM), a well known

Table 1. Caspase-3 Activity in the Presence of Euryfurylnaphthoquinones and Sanguinarine

	No addition	+Vitamin C (2 mM)
	(mUnits)	
Control	15.0±2.0	16.7±1.6
$\mathbf{C}_1$	12.8±1.2	13.9±1.8
$\mathbf{C}_2$	13.2±1.9	15.5±2.4
$\mathbf{C}_3$	18.2±2.1	19.3±1.6
Sanguinarine (10 μM)	88.9±7.5*	Not determined

TLT cells were incubated for 4 h at 37 °C with euryfurylnaphthoquinones ($\mathbf{C}_1$, $\mathbf{C}_2$ and $\mathbf{C}_3$) at 5 μg/ml, in the absence and in the presence of 2 mM vitamin C. Aliquots of cell suspension were taken and the activity of caspase-3 was measured as indicated in Experimental. The results are expressed as mUnits and represent the mean values±S.E.M. of three different experiments. * $p<0.05$ as compared to control conditions.

inducer of apoptosis *via* caspase-3 activation,[9] provokes an increase by 6-fold in the activity of the enzyme. Indeed, after 4 h of incubation, the activity was of 88.9 mUnits as compared to 15.0 mUnits observed under control conditions.

Chemistry The electrochemical behaviour of quinonoid compounds is very important to understand their bioreductive activation process. The metabolism of quinone-containing antitumor agents involves enzymatic reduction of the quinone by one or two electrons. This reduction results in the formation of the semiquinone or the hydroquinone of the anticancer drug. The consequence of these enzymatic reductions is that the semiquinone yields its extra electron to oxygen with the formation of superoxide radical anion and the original quinone. The formation of superoxide radical anion is the beginning of a cascade that generates H_2O_2 and hydroxyl radicals (OH), generally referred to as reactive oxygen species (ROS). This reduction by a reductase followed by oxidation by molecular oxygen is known as redox-cycling and continues until the system becomes anaerobic. When two electrons are involved in the reduction of the quinone the corresponding hydroquinone could become stable and is excreted by the organism in a detoxification pathway.[15—18]

Taking into account that quinones $\mathbf{C}_1$, $\mathbf{C}_2$ and $\mathbf{C}_3$ have an euryfuryl substituent at the 2-position, the potential reductive activation processes of these quinones should be mainly determined by the electrochemical properties of their corresponding quinone moieties which are structurally related to the parent quinones **2**, **3** and **4**. The half-wave potentials $E_{1/2}$ due to the addition of the first electron to the naphthoquinones **2**, **3** and **4,** which were reported in the literature, indicate that the introduction of a hydroxyl group in the 5-position of **2** changed the $E_{1/2}$ value of the parent quinone **2** from −556 to −405 mV (dimethyl sulfoxide (DMSO)). The magnitude of the observed negative change indicates that the effect of one hydrogen bond in **3** overcomes the electron donor effect of the hydroxy group thus increasing the electron-acceptor capability of the quinone nucleus with respect to quinone **2**. Interestingly, the introduction of two hydroxyl groups in the 5- and 8-position in quinone **2** changed the $E_{1/2}$ potential of quinone **2** from −556 to −312 mV (DMSO).[19]

These data indicate that the introduction of hydroxyl groups in the *peri*-positions of quinone **2**, as in quinones **3** and **4**, increases the electrophilicity of the quinoid nucleus as compared to the parent quinone **2** probably due to hydrogen bond interactions, making them a sort of an internal Bröensted acid.

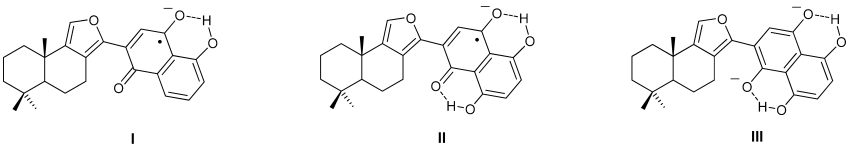

Fig. 3. Structures of the Radical Anion Semiquinones and Hydroquinone Dianion Generated from $\mathbf{C}_2$ and $\mathbf{C}_3$

Based on the electrochemical behaviour of quinones **3** and **4** and on the hydrogen bond stabilization of their corresponding reductive species I, II and III (Fig. 3), it appears that the formation of I is more favourable than II. Indeed, the stability of II is lower than I because it accepts a second electron to give the high hydrogen bond species III. Therefore $\mathbf{C}_2$ should be a better redox-cycler than $\mathbf{C}_3$ leading to ROS formation and the subsequent cell death by oxidative stress.[19)]

Discussion

Due to oncogenic stimulation and high metabolic rates, cancer cells usually exhibit high levels of reactive oxygen species (ROS) that stimulate cell proliferation and promote genetic instability.[20,21)] Such a biochemical difference between transformed and non-transformed cells, renders cancer cells particularly vulnerable towards any treatment interfering with the redox homeostasis. Accordingly, it has recently been reported that inhibition of antioxidant defences leads to a ROS accumulation that affects mainly cancer cells.[22)] In addition, several reports show an impairment of antioxidant enzymes during carcinogenesis.[4,5,23)]

Several data support the involvement of ROS in the cytolytic action of anticancer treatments. Indeed, the efficacy of radiotherapy is well-known to rely on the presence of oxygen—the so-called oxygen effect—and an elevation of intracellular ROS is involved in the cytotoxicity of many chemotherapeutic agents.[24—26)] Confirming this hypothesis, several *in vitro* studies show that the antioxidant capacities of tumour cells are linked to their resistance towards chemotherapy[27,28)] and radiotherapy.[29—31)] Thus, tumours that exhibit low antioxidant enzyme levels are associated with a better response to radiotherapy.[32,33)] In view of these data, we postulate that the inhibition of the antioxidant capacities of tumours would result in the potentiation of anticancer treatments by increasing the intracellular formation of ROS.

The results reported here indicate that compounds $\mathbf{C}_1$ and $\mathbf{C}_3$ are non toxic against TLT cells, while compound $\mathbf{C}_2$ induces a strong cytotoxic effect on these cells. It should be underlined that $\mathbf{C}_2$ also exhibited higher cytotoxic activity than $\mathbf{C}_1$ and $\mathbf{C}_3$ against *Leishmania amazonensis*.[14)] So far, such a cell death seems to be rather necrotic since caspase-3 activation, a hallmark of apoptosis, was not observed with any of the three compounds tested. Interestingly, the presence of vitamin C did not induce a putative activation of the cytotoxic damage, in terms of cell death. However, its addition exacerbates the effects of three compounds on both ATP and GSH content, the two metabolic end points we selected in our study.

In conclusion, we have demonstrated that insertion of hydroxyl groups in the *peri* position of 2-euryfuryl-1,4-naphthoquinone $\mathbf{C}_1$ influences the capacity of the electroactive group to undergo a reductive process and thus modify the electron transfer from ascorbate to the electroactive quinone nucleus. Quinone $\mathbf{C}_2$ induces a necrotic cell death even in the absence of vitamin C. However, the combination of these quinones with vitamin C provokes a strong impairment in cellular homeostasis as shown by a decease of both ATP and GSH contents. This mechanism is of potential clinical interest because cancer cells take up vitamin C, they are sensitive to an oxidant insult and they depend on glycolysis (ATP formation) for their survival.

Experimental

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue,[34)] 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 Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) according to procedures described by the suppliers, and the results are expressed as nmol ATP/mg proteins. The protein content was determined by the method of Lowry *et al.*[35)] using bovine serum albumin (BSA) as reference. Reduced gluthatione (GSH) was measured according to the *ortho*-phtalaldehyde (oPT) method as reported elsewhere[36)] and the results are expressed as nmol GSH/mg proteins. 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 (Biorad). The results are expressed as mUnits.

Data were analysed 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 analysed using Student's *t* test. The level of significance was set at $p<0.05$.

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