Differential effects of Bcl-2 and caspases on mitochondrial permeabilization during endogenous or exogenous reactive oxygen species-induced cell death

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Title: Differential effects of Bcl-2 and caspases on mitochondrial permeabilization during endogenous or exogenous reactive oxygen species induced cell death

Subtitle: A comparative study of H_2O_2, paraquat, *t*-BHP, etoposide and TNF-α induced cell death

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Abstract: In this study, we have compared several features of cell death triggered by classical inducers of apoptotic pathways (etoposide and TNF-α) versus exogenous ROS (H_2O_2, *tert*-butyl hydroperoxide (*t*-BHP)) or a ROS generator (paraquat). Our aim was to characterize the relationships that exist between ROS, mitochondrial perturbations, Bcl-2 and caspases, depending on the source and identity of ROS. First, we have found that these five inducers trigger oxidative stress, mitochondrial membrane permeabilization (MMP) and cell death. In each case, cell death could be inhibited by several antioxidants, showing that it is primarily ROS-dependent. Second, we have evidenced that during etoposide or TNF-α treatments, ROS accumulation, MMP and cell death are all regulated by caspases and Bcl-2, with caspases acting early in the process. Third, we have demonstrated that H_2O_2-induced cell death shares many of these characteristics with etoposide and TNF-α, whereas *t*-BHP induces a caspase-dependent cell death with caspases acting downstream of the process. Finally, we have observed that paraquat triggers both MMP and cell death which are Bcl-2 and caspase-independent. On the one hand, these results show that endogenous or exogenous ROS can trigger multiple cell death pathways with Bcl-2 and caspases acting differentially. On the other hand, they suggest that H_2O_2 could be an important mediator of etoposide and TNF-α since these inducers trigger similar phenotypes.

Abbreviations: ΔΨm: mitochondrial membrane potential; eto, etoposide; E/TNF, emetine plus TNF-α; MMP, mitochondrial membranes permeabilization; PI, propidium iodide; ROS, reactive oxygen species; *t*-BHP, *tert*-butyl hydroperoxide; TNF, tumor necrosis factor; Vit E, vitamin E.

Introduction

Reactive oxygen species (ROS) are a family of highly reactive molecules that includes hydroxyl radical ($HO^{\cdot}$), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and organic peroxide radicals. Under physiological conditions, the mitochondrial respiratory chain is the major site for ROS production in cells (Fleury et al. 2002). ROS are extremely transient species due to their high chemical reactivity that is responsible for their destructiveness on DNA, proteins, carbohydrates and lipids. It is now admitted that ROS are important mediators of several types of cell death such as apoptosis and necrosis. Apoptosis is mediated by a family of cystein proteases known as caspases. In mammals, there are two main pathways by which caspase activation is triggered: the intrinsic and extrinsic apoptotic pathways. Various signals that trigger the intrinsic pathway (such as oxidative or genotoxic stress) are mainly transduced to the mitochondria which then undergo a series of biochemical events resulting in the mitochondrial membranes permeabilization (MMP) (Jourdain and Martinou 2009; Kroemer et al. 2007) and the release of pro-apoptotic molecules from mitochondria, such as cytochrome c (Li et al. 1997). The extrinsic pathway is activated by the binding of ligands, such as FasL or TNF-α, to their receptors on the cell surface that can directly activate caspases (Thorburn 2004). This pathway may also require the involvement of mitochondria, notably through the caspase-dependent production of the pro-apoptotic protein tBid (Li et al. 1998). The mitochondrial pathway is regulated by members of the Bcl-2 family, which includes both anti-apoptotic proteins (such as Bcl-2) and pro-apoptotic proteins (such as Bax) which respectively repress or stimulate MMP (Desagher and Martinou 2000; Wang and Youle 2009).

A ROS-dependent cell death can be triggered by classical apoptosis inducers such as etoposide and TNF-α, which respectively trigger the intrinsic and the extrinsic pathways of

cell death. We have previously shown in HeLa cells that TNF-α and etoposide-induced apoptosis involves early mitochondrial ROS production that strongly accelerates the process of cell death (Dumay et al. 2006; Sidoti-de Fraisse et al. 1998). Etoposide (eto) is a topoisomerase II inhibitor, which triggers the intrinsic caspase activation pathway, involving mitochondrial perturbations (Karpinich et al. 2002), whereas TNF-α is a cytokine that triggers the extrinsic caspase activation pathway but does not necessarily involve mitochondria (Thorburn 2004). We have also used exogenous ROS or ROS generators to induce cell death in HeLa cells by incubating them with hydrogen peroxide (H_2O_2), paraquat and *tert*-butyl hydroperoxide (*t*-BHP). Among ROS, H_2O_2 represents a particularly important molecule because it is generated under nearly all oxidative stress conditions and it can participate in several fundamental intracellular processes. Paraquat is a non-selective herbicide which undergoes redox cycling *in vivo*, being reduced by an electron donor such as NADPH, before being oxidized by an electron receptor such as O_2 to produce superoxide. Next, *t*-BHP is a short chain analog of lipid hydroperoxides which mimics the toxic effect of peroxidized fatty acids.

In this study, we compared several features of cell death triggered by etoposide and TNF-α versus the pro-oxidant molecules H_2O_2, *t*-BHP and paraquat. Our aim was to characterize the relationships that exist between ROS, mitochondrial perturbations, Bcl-2 and caspases, depending on the origin and identity of ROS.

Materials and methods

Reagents

α-tocopherol (vitamin E), Butylated hydroxyanisole (BHA), catalase, diphenylene iodonium chloride (DPI), emetine (E), etoposide (eto), hydrogen peroxide solution (H_2O_2), methyl viologen dichloride hydrate (paraquat), N-acetyl-cysteine (NAC), Noridihydroguaiaretic acid (NDGA), propidium iodide (PI), 1,2-dihydroxybenzene-3,5-disulfonic acid (tiron), mouse tumour necrosis factor-α (TNF-α) and tert-butyl hydroperoxide solution (*t*-BHP) were from Sigma. 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and 3,3'-diethyloxacarbocyanine ($DiOC_6(3)$) were from Invitrogen. z-Val-Ala-DL-Asp-Fluoromethylketone (zVAD-fmk) and N-Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO) were from Bachem. Stock solutions were prepared as follows: NAC (50 mM), PI (1 mg/ml) and tiron (1 M) in water; catalase (10^5 U/ml), emetine (1 mg/ml), paraquat (100 mM), and TNF-α (1 μg/ml) in serum free medium; BHA (200 mM), DCFH-DA (20 mM), $DiOC_6(3)$ (1 mM), NDGA (40 mM) and Vit E (100 mM) in ethanol; DPI (3 mM) and etoposide (25 mg/ml) in dimethyl sulfoxide (DMSO); zVAD-fmk (50 mM) and DEVD-CHO (100 mM) in methanol. All stock solutions were stored at –20°C.

Cell lines, cell culture and induction of cell death

HeLa and HeLa-Bcl-2 cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum together with penicillin (100 μg/ml), streptomycin (100 U/ml) and glutamax (1% v/v) from Invitrogen. The HeLa-Bcl-2 cell line contains the human *bcl-2* cDNA under

control of the Tet Off inducible expression system (Clontech). These cells were initially cultured in the presence of tetracycline (2 μg/ml) to inhibit the expression of exogenous Bcl-2 and then propagated for 10 days without tetracycline to allow the accumulation of the Bcl-2 protein prior the experiments. Cell death was induced by the addition on exponentially growing adherent cells of H_2O_2 (200 μM), paraquat (5 mM), *t*-BHP (200 μM), etoposide (20 μg/ml) or TNF-α (2.5 ng/ml) plus emetine (1 μg/ml). After a 24 h incubation, cells were analyzed by flow cytometry. Antioxidants (BHA (300 μM), catalase (1000 units/ml), DPI (2.5 μM), NAC (5 mM), NDGA (40 μM), tiron (1 mM), Vit E (100 μM)) or caspase inhibitors: DEVD-CHO (300 μM), zVAD-fmk (50 μM), were added at the same time than cell death inducers.

Flow cytometry

Flow cytometric measurements were performed using a XL3C flow cytometer (Beckman-Coulter). Fluorescence was induced by the blue line of an argon ion laser (488 nm) at 15 mW. Green and red fluorescence were respectively collected with 525 nm and 620 nm band pass filters. Analyses were performed on 10^4 cells. The mitochondrial membrane potential ($\Delta\Psi m$) was assessed by the retention of the green fluorescent $DiOC_6(3)$. This cationic lipophilic fluorochrome is a cell permeable marker that, at low doses, specifically accumulates in mitochondria depending on the mitochondrial membrane potential (Petit et al. 1990). Intracellular ROS level was measured by the production of dichlorofluorescein (DCF) derived from oxidization of DCFH. The non fluorescent DCFH-DA is a freely permeable tracer which is deacylated by intracellular esterases to the nonfluorescent compound DCFH and oxidized to the green fluorescent compound DCF by a variety of peroxides, including hydrogen peroxide (Ubezio and Civoli 1994). The red fluorescent PI specifically penetrates in cells which have

lost their plasma membrane integrity and was therefore used to characterize necrotic cells (Darzynkiewicz et al. 1994). Finally, the decrease in forward scatter that reflects cell shrinkage during the last steps of cell death was also assessed (Dive et al. 1992). The percentage of non necrotic cell death was calculated as the percentage of cell shrinkage in the PI negative population. After drug treatment, the media from the culture dishes (containing late apoptotic cells) were kept in centrifuge tubes. The adherent cells (containing living and early apoptotic cells) were detached using trypsine and pooled with the corresponding media, centrifuged and resuspended in complete medium at a concentration of $1x10^6$ cells/ml. Cells were then loaded with 0.1 μM $DiOC_6(3)$ or 20 μM DCFH-DA for 30 min at 37°C and incubated with 10 μg/ml PI, 5 min prior analysis.

Statistical analysis

Statistical analysis was determined using Student's *t*-test. $P<0.05$ was accepted as significant.

Results

The apoptosis inducers etoposide and TNF-α, the exogenous ROS H_2O_2 and *t*-BHP, as well as the ROS generator paraquat all trigger intracellular ROS accumulation, mitochondrial membrane permeabilization and cell death

We have previously shown that etoposide and TNF-α trigger endogenous mitochondrial ROS production in HeLa cells (Dumay et al. 2006; Sidoti-de Fraisse et al. 1998) whereas H_2O_2, paraquat and *t*-BHP are often used as inducers of oxidative stress and cell death. TNF-α used alone is generally not sufficient to kill cultured cells since the interaction between TNF-α and its receptors induces antagonistic signals: the formation of the Death Inducing Signaling Complex (DISC), which rapidly activates caspases, and the expression of endogenous caspase inhibitors (IAPs) through the activation of NF-κB (Nagata 1997). Thus, TNF-α is currently used *in vitro* in combination with a protein synthesis inhibitor such as emetine (E) which is able to inhibit the NF-κB-dependent expression of caspase inhibitors, without interfering with DISC formation.

Since we wanted to test the influence of ROS on mitochondrial perturbations, we started to determine concentrations of the different compounds (etoposide (eto), emetine plus TNF-α (E/TNF), H_2O_2, paraquat and *t*-BHP) that induce a significant drop in ΔΨm. Fig. 1A shows that after a 24h incubation (concentrations indicated in figure legend), approximately one half of the cells exhibits a low ΔΨm, indicating that they have undergone a permeabilization of their mitochondrial membranes (MMP). These conditions were considered as ideal to study inhibitory or promoting effects on MMP. Next, we have measured the percentage of cells harboring high ROS levels, corresponding to the cells undergoing oxidative stress. Fig. 1B indicates that etoposide, E/TNF and paraquat can trigger the

accumulation of endogenous ROS and that the exogenous H_2O_2 and *t*-BHP also provoke an efficient, but probably more direct, oxidative stress. The accumulation of ROS was measured by using DCFH-DA, a probe which becomes fluorescent after reacting with peroxides. Since peroxides are classical by-products of oxidant molecules, DCFH-DA is a good probe to detect oxidative stress from different origins. However, it must be kept in mind that its modification requires a deacetylation process prior oxidation, which cannot be performed in late dead cells with inactive esterases, such as in population (c) (see Fig. 1B). Thus, cells undergoing oxidative stress are always underestimated with this probe when cell death occurs. Next, we were also interested in the events that could occur downstream of mitochondrial perturbations (such as cell death) and we have characterized two additional parameters by flow cytometry. The first parameter is the percentage of propidium iodide (PI) positive / necrotic cells (Fig. 1C, population (c)). The second one is the percentage of PI negative cells with reduced size (cell shrinkage) which indicates the percentage of late apoptotic (or autophagic) cell death (Fig 1C, population (b)). Our results show that all the inducers trigger both necrotic and non necrotic cell death. The fact that specific inducers of apoptosis (etoposide, E/TNF) also trigger necrosis may be surprising. However, it is well known that a secondary necrotic process provoked by the lack of engulfment of dying cells can occur *in vitro*.

Etoposide, E/TNF, H_2O_2, paraquat and *t*-BHP all induce a ROS dependent cell death which can be inhibited by several antioxidants

Although all inducers seemed to trigger an oxidative stress, MMP and cell death (see Fig.1), the pathways involved could be different from one inducer to another. Thus, we wanted to determine, in each case, what is the causal link between ROS accumulation and these events. For this purpose, we have incubated cells with cell death inducers and antioxidants at the

same time. Here, we have used 7 antioxidants with distinct properties (see materials and methods for a complete name of the compounds): BHA is a food additive (E320) which has also been shown to inhibit mitochondrial respiration (Aldunate et al. 1992); catalase is a well known enzyme which converts H_2O_2 into H_2O and O_2; DPI is a NADPH oxidase inhibitor (Meier et al. 1991); NAC is a precursor to glutathione, a natural antioxidant protein; NDGA is a cyclooxygenase and lipoxygenase inhibitor (Van Wauwe and Goossens 1983); tiron is a superoxide scavenger (Greenstock and Miller 1975) and vitamin E is an inhibitor of lipid peroxidation (Tappel 1970). We have quantified cell death (as the percentage of cell shrinkage) in presence or in absence of the antioxidant, and calculated the percentage of cell death inhibition. The results presented in Fig. 2 show that the effect of each inducer is inhibited by at least three different antioxidants, showing that cell death is tightly linked to oxidative stress. Some results were expected: catalase strongly inhibits H_2O_2-induced cell death; DPI strongly inhibits the effect of paraquat (mediated by NADPH oxidases); vitamin E inhibits toxicity induced by *t*-BHP (an analog of lipid hydroperoxides) and BHA inhibits the mitochondrial-dependent production of ROS induced by etoposide and E/TNF. Other results are more surprising and would deserve additional investigations. For instance, it is astonishing that tiron does not inhibit the effect of paraquat which is known to generate superoxide, whereas the effect of *t*-BHP, a molecule unrelated to superoxide, is strongly inhibited by tiron. Further studies using other ROS-sensitive probes should help to clarify these results.

Influence of Bcl-2 on MMP, ROS accumulation and cell death

In order to test the effect of the anti-apoptotic protein Bcl-2 on the phenotypes triggered by the five inducers, we have used the HeLa-Bcl-2 cell line which can overexpress the human *bcl-2* gene under control of tetracycline (Tet Off system from Clontech). With this system, the

absence of tetracycline in the culture medium leads to the accumulation of Bcl-2. The results presented in Fig. 3A show that MMP is significantly inhibited in all cases except for paraquat, demonstrating that the mechanism of MMP triggered by paraquat is different from the other inducers. Next, we can see in Fig. 3B that Bcl-2 significantly inhibits the accumulation of cells with high ROS levels with H_2O_2, *t*-BHP, etoposide and E/TNF. This result is interesting for several reasons: first, since etoposide and E/TNF trigger the production of endogenous mitochondrial ROS (we have previously shown that this production is severely reduced in HeLa ρ^0 cells, demonstrating their mitochondrial origin (Sidoti-de Fraisse et al. 1998)), it indicates that Bcl-2 can act on the mitochondrial production of ROS. In this case, Bcl-2 may directly or indirectly regulate the mitochondrial respiration chain. Second, the fact that Bcl-2 inhibits oxidative stress after exposition to exogenous ROS suggests that Bcl-2 can also act as an antioxidant. Such functions for Bcl-2 have already been described but are still poorly understood (Hockenbery et al. 1993; Vrbacky et al. 2003). Finally, Bcl-2 does not seem to inhibit ROS accumulation induced by paraquat. Thus, it is possible that Bcl-2 negatively regulates mitochondrial superoxide production and/or exogenous peroxides accumulation while being unable to act on cytosolic superoxide, produced by enzymes such as NADPH oxidase. Concerning the regulation of cell death, it appears from Fig. 3C that Bcl-2 is both an inhibitor of necrotic and non necrotic cell death, except for paraquat-induced cell death which is apparently totally disconnected from any Bcl-2 regulation. We can hypothesize that Bcl-2 protects against H_2O_2, *t*-BHP, etoposide and E/TNF-induced necrosis because it is a secondary necrotic process (due to a lack of engulfment of dying cells), while necrosis observed during paraquat-induced cell death would be primary necrosis.

Influence of caspase inhibition on MMP, ROS accumulation and cell death

Here we have used two chemical inhibitors to determine the role of initiator and executive caspases: zVAD (Z) which is an inhibitor of both initiator and executive caspases as well as DEVD (D) which is a specific inhibitor of the executive caspases -3 and -7. Fig. 4A shows that zVAD significantly decreases MMP during H_2O_2, etoposide and E/TNF-induced cell death, indicating that caspases regulate MMP, acting upstream of or at mitochondria. The effect of DEVD on etoposide and E/TNF is significant ($p<0.05$), whereas it is nearly significant for H_2O_2 ($p=0.059$). Concerning paraquat and *t*-BHP-induced cell death, it appears that MMP is not influenced by the presence of zVAD or DEVD, demonstrating that this process is caspase-independent and necessarily distinct from the process observed for H_2O_2, etoposide and E/TNF. Caspase-inhibitors were also found to influence the accumulation of cells with high ROS levels (Fig. 4B) for H_2O_2, etoposide and E/TNF. zVAD totally inhibits ROS accumulation with etoposide and E/TNF, suggesting that endogenous ROS production first requires the activation of caspases. The milder effect of DEVD was found to be significant for etoposide but not for E/TNF ($p=0.067$). The fact that zVAD inhibits endogenous ROS production in E/TNF cell death was expected since DISC formation and activation of initiator caspases is one on the first events following the interaction between TNF-α and its receptors. The results that were found for etoposide also suggest that a caspase, acting upstream of or at mitochondria, regulates ROS accumulation and MMP. Concerning H_2O_2, the fact that zVAD and DEVD significantly increase the percentage of cells with high ROS levels is even more surprising. One possibility is that caspase-inhibition would increase the resistance of the cells to (exogenous) H_2O_2, therefore delaying their disappearance. This result would necessarily be different from etoposide and TNF-α where zVAD and DEVD also inhibit (endogenous) ROS accumulation. In contrast to H_2O_2, etoposide and E/TNF, caspase inhibitors do not modify MMP or ROS accumulation during paraquat or *t*-BHP treatments, indicating that these processes are caspase-independent. Next, Fig. 4C shows that caspase

inhibitors attenuate necrotic cell death for H_2O_2, etoposide and E/TNF (but not for paraquat and *t*-BHP), suggesting that secondary necrosis occurs with these three inducers. The caspase inhibitors also diminish non necrotic cell death for all the inducers except paraquat. In conclusion, these results indicate that paraquat-induced cell death is caspase-independent, and that *t*-BHP-induced necrosis is caspase-independent whereas it was Bcl-2-regulated. Since some studies report that Bcl-2 can inhibit necrosis (Guenal et al. 1997; Kane et al. 1995), *t*-BHP may also induce primary necrosis.

Discussion

Here we have shown that H_2O_2, paraquat, *t*-BHP, etoposide and E/TNF are all able to induce a ROS-dependent MMP and cell death, but that the mechanisms underlying these effects can be different from one inducer to another. Etoposide and E/TNF induce the most similar phenotypes despite the fact that they have quite different targets. For both inducers, our results suggest that a caspase activity is involved upstream of mitochondrial ROS production and MMP since zVAD totally inhibits these processes (Fig. 4). While this is not surprising for E/TNF, because caspases are activated very early during the intrinsic pathway of apoptosis, this result was unexpected for etoposide. However, we have previously shown that zVAD inhibits cytochrome c release in HeLa cells treated with E/TNF whereas it is not the case for etoposide (Dumay et al. 2006). This suggests that contrary to E/TNF, a first wave of cytochrome c release would occur before ROS overproduction and MMP during etoposide-induced cell death. A feedback loop linking caspases to mitochondria would then amplify mitochondrial perturbations (Chen et al. 2000). This feedback loop can be evidenced for both E/TNF and etoposide by the effect of DEVD on MMP and ROS accumulation (Fig. 4).

H_2O_2 is the inducer that triggers the closest phenotype to etoposide and E/TNF, suggesting that this molecule could be an important mediator of etoposide and E/TNF-induced cell death. Interestingly, it can be noticed that contrary to etoposide and E/TNF, zVAD cannot totally inhibit the drop of $\Delta\Psi m$ induced by H_2O_2 (Fig. 4), suggesting that H_2O_2 can trigger MMP prior caspase activation. In this case, the effect of zVAD would be associated with the inhibition of the caspase-dependent feedback loop on mitochondria. Next, *t*-BHP, which is also a peroxide, mainly differs from H_2O_2 by the fact that it triggers a completely caspase-independent $\Delta\Psi m$ decrease (Fig. 4). The mechanisms involved in mitochondrial membranes permeabilization is still controversial and two main models have been proposed, which may

both exist, depending on death signals and the cellular context (Desagher and Martinou 2000; Kroemer et al. 2007). The first type of models relies on the formation in the outer mitochondrial membrane of large channels constituted of pro-apoptotic members of the Bcl-2 family (such as Bax) which leads to the release of intermembrane space proteins including cytochrome c whose depletion leads to $\Delta\Psi m$ loss. Alternatively, a deregulation of the permeability transition pore complex (PTP) located at the outer/inner membrane junctions and constituted of the porin VDAC and the adenine nucleotide translocase (ANT), can also lead to $\Delta\Psi m$ loss and outer membrane permeabilization. Thus it is possible that the regulation of MMP between H_2O_2 (Bcl-2 and caspase-dependent), *t*-BHP (Bcl-2-dependent and caspase-independent) and paraquat (Bcl-2 and caspase-independent) involves the formation of distinct pores having different sensitivities to caspases and Bcl-2. Interestingly, it has been shown in HepG2 cells that *t*-BHP triggers an hyperpolarization preceding a loss of $\Delta\Psi m$, a result which is reminiscent of a model that involves VDAC closure and swelling of mitochondria (Desagher and Martinou 2000). Little is known about the influence of paraquat on MMP (Costantini et al. 1995; Palmeira et al. 1995) whereas etoposide- and E/TNF-induced MMP seems to involve pro-apoptotic members of the Bcl-2 family (Dumay et al. 2006; Li et al. 1998).

Finally, we have shown that H_2O_2, paraquat, *t*-BHP, etoposide and E/TNF all trigger necrotic and non necrotic cell death. While it seems that H_2O_2, etoposide and E/TNF trigger secondary necrosis, it would rather be a primary necrosis for *t*-BHP and paraquat. Surprisingly, paraquat is able to induce a caspase-independent and non necrotic cell death in approximately half of the treated cells, as assessed by the percentage of non necrotic cells undergoing cell shrinkage (Fig. 1). It would be interesting to understand what type of alternative cell death is induced by paraquat and how its execution can be managed independently from caspases.

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Legends to figures

Fig. 1 Effects of apoptosis inducers and exogenous ROS on (A) mitochondrial membrane potential (ΔΨm), (B) intracellular ROS level and (C) cell death. HeLa cells were incubated with the following drugs: H_2O_2 (200 μM), paraquat (5 mM), *t*-BHP (200 μM), etoposide (20 μg/ml), emetine (1 μg/ml) plus TNF-α (2.5 ng/ml), during 24 h and analyzed by flow cytometry (see materials and methods). Typical cytograms are indicated on the right panel (control versus etoposide) for each labeling. (A) During $DiOC_6(3)$ fluorescence analysis, cells with high (a) and low (b) fluorescence were distinguished. The percentages of cells with low ΔΨm (b) are indicated in the histogram on the left panel. (B) Concerning intracellular ROS measurements (DCF fluorescence), normal cells (a), cells with high ROS levels (b) and late dead cells which cannot produce DCF fluorescence were distinguished (c). The percentages of cells with increased intracellular ROS levels (b) are indicated in the histogram on the left panel. (C) For PI labeling, normal cells (a) were distinguished from non necrotic dead cells (b) (reduced forward scatter and low PI fluorescence) and from necrotic cells (c) (reduced forward scatter and increased PI fluorescence). The total percentages of cell death are indicated in the histogram on the left panel as the sum of necrotic and non necrotic cell death.

Fig. 2 Inhibition of etoposide, E/TNF, H_2O_2, paraquat and *t*-BHP-induced cell death by antioxidants. HeLa cells incubated with the five inducers were treated at the same time with antioxidants: BHA (300 μM), catalase (1000 units/ml), DPI (2.5 μM), NAC (5 mM), NDGA (40 μM), tiron (1 mM), Vit E (100 μM). Cells were analyzed by flow cytometry 24 h after beginning of the treatment. Overall cell death was measured by quantifying the cells that harbored small cell size (cell shrinkage). The percentage of cell death inhibition was calculated from values obtained with cells treated and untreated with a specific antioxidant.

The absence of a bar for several cell death inducer / antioxidant combinations indicates that no protection was found in these particular cases.

Fig. 3 Regulation of etoposide, E/TNF, H_2O_2, paraquat and *t*-BHP-induced cell death by Bcl-2. HeLa-Bcl-2 cells overexpressing (+Bcl-2) or not (-Bcl-2) the anti-apoptotic protein Bcl-2 were used in this study (see materials and methods). These cells were treated with the same concentrations of cell death inducers than for Fig. 1: H_2O_2 (200 μM), paraquat (5 mM), *t*-BHP (200 μM), etoposide (20 μg/ml), emetine (1 μg/ml) plus TNF-α (2.5 ng/ml), during 24 h, and were analyzed by flow cytometry. The histograms indicate the percentages of cells with low ΔΨm (A), high ROS levels (B) as well as cell death (C). * Statistically significant differences with the same treatment between cells overexpressing (+ Bcl-2) or not Bcl-2 (- Bcl-2) are also indicated. In (C), each significant difference was found for both necrotic and non necrotic cell death.

Fig. 4 Inhibition of etoposide, E/TNF, H_2O_2, paraquat and *t*-BHP-induced cell death by caspase inhibitors. HeLa cells incubated with the five inducers were treated at the same time with caspase inhibitors: the broad spectrum caspase inhibitor zVAD-fmk (Z) which inhibits both initiator and executive caspases or DEVD-CHO (D) which specifically inhibits executive caspases. zVAD-fmk and DEVD-CHO were respectively used at 50 μM and 300 μM. Cells were analyzed by flow cytometry 24 h after beginning of treatment. The histograms indicate the percentages of cells with low ΔΨm (A), high ROS levels (B) as well as cell death (C). * Statistically significant differences between control and zVAD or DEVD are also indicated. In (C), Δ indicates that a significant difference was found for non necrotic cell death, but not for necrotic cell death.

Figure 1

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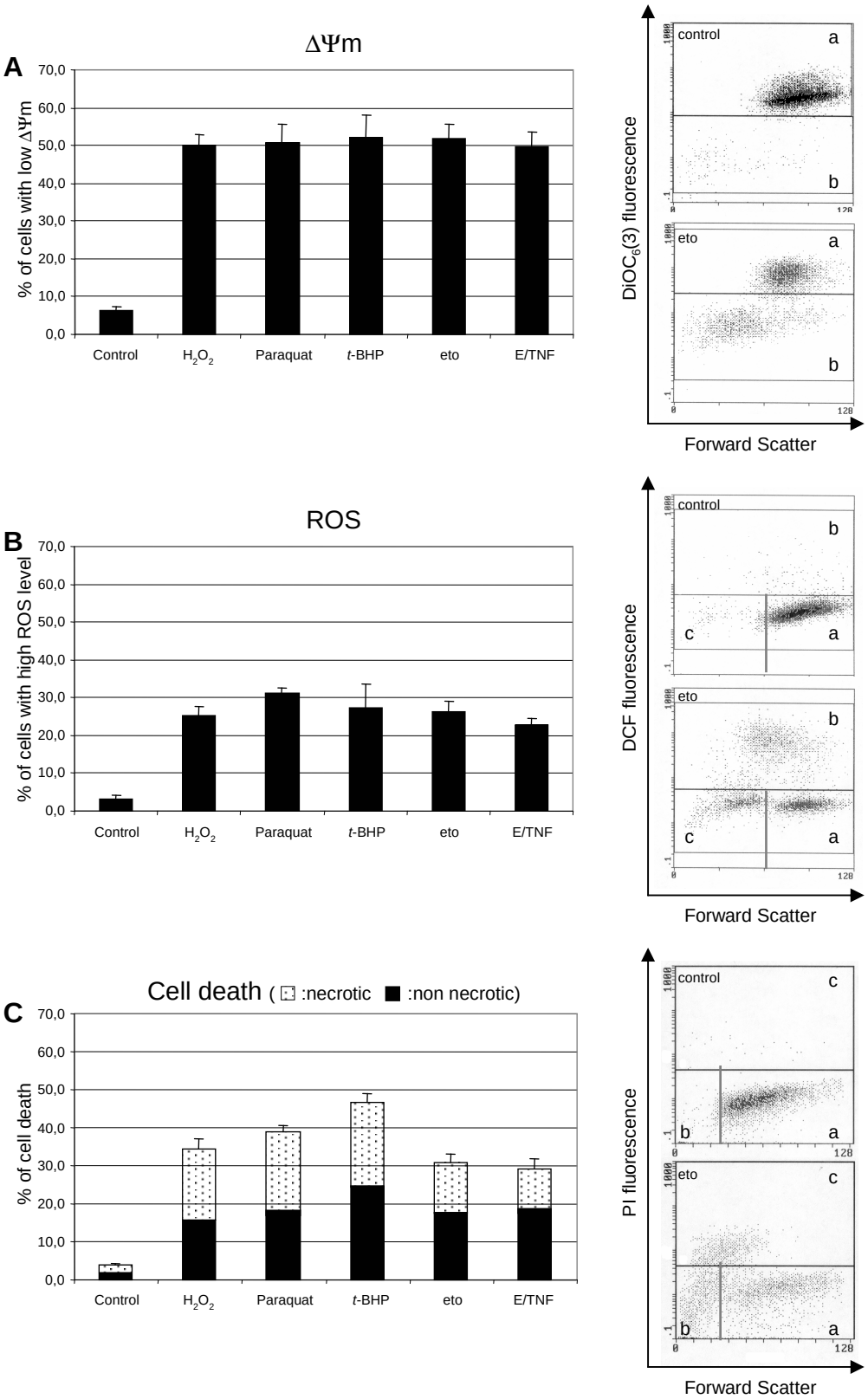

Figure 1

Figure 2

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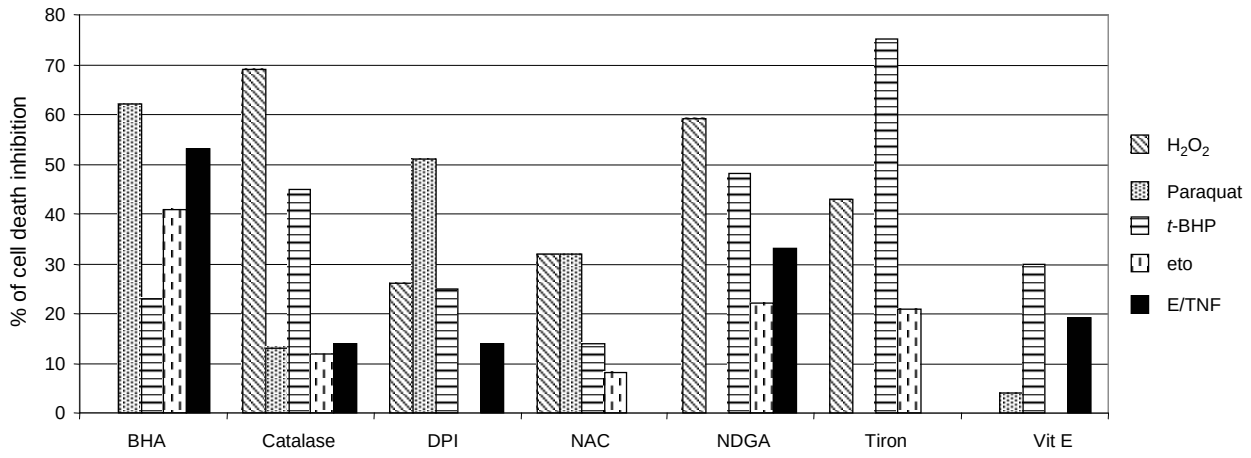

Figure 2

Figure 3

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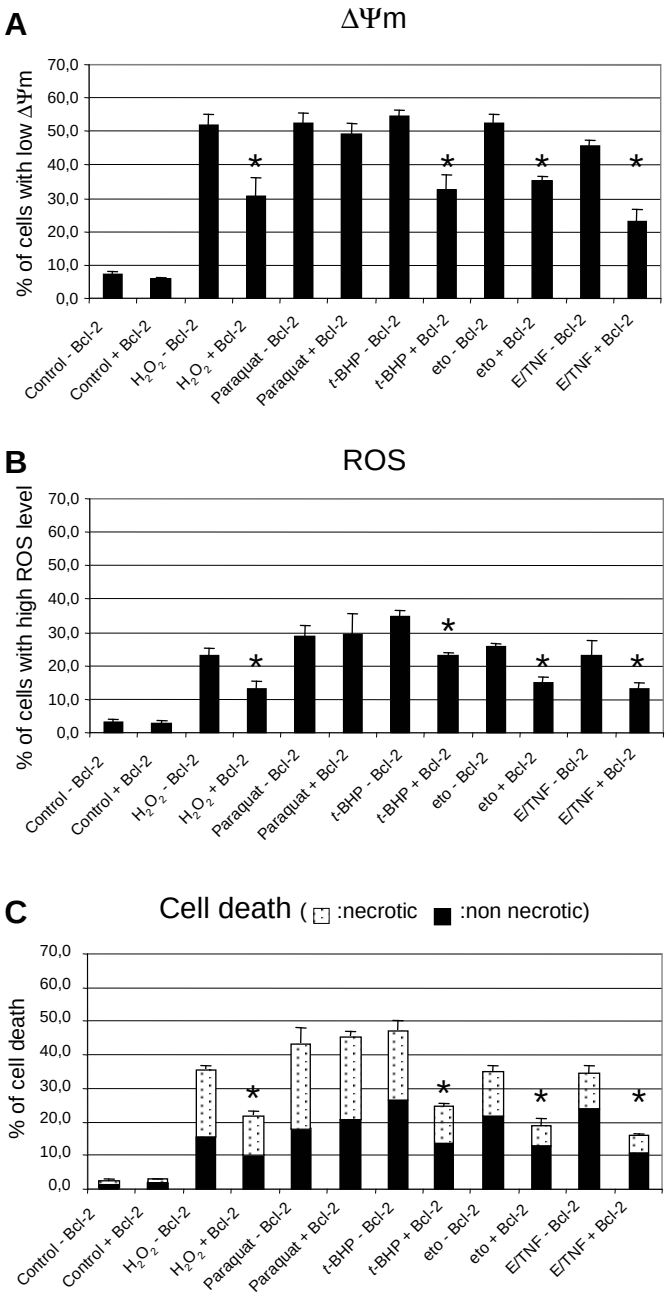

Figure 3

Figure 4

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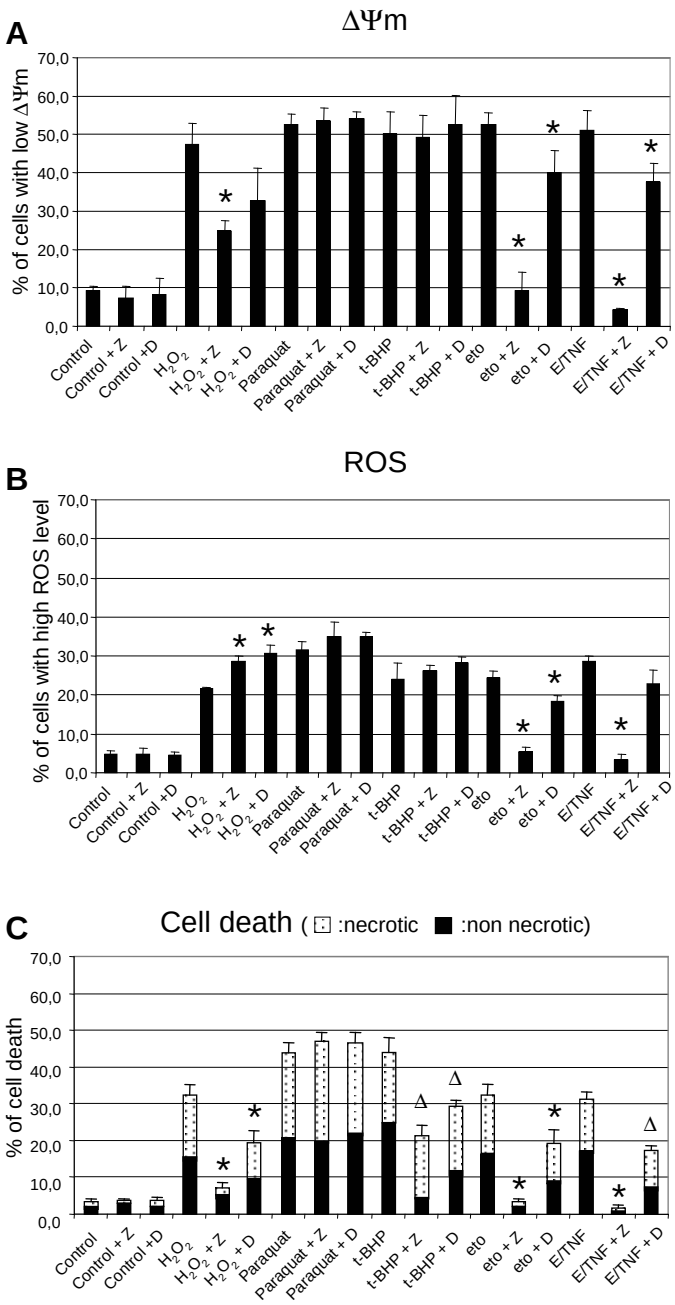

Figure 4