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Intracellular ATP levels determine cell death fate of cancer cells exposed to both standard and redox chemotherapeutic agents

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ABSTRACT

Cancer cells generally exhibit high levels of reactive oxygen species (ROS) that stimulate cell proliferation and promote genetic instability. Since this biochemical difference between normal and cancer cells represents a specific vulnerability that can be selectively targeted for cancer therapy, various ROS-generating agents are currently in clinical trials, either as single agents or in combination with standard therapy. However, little is known about the potential consequences of an increased oxidative stress for the efficacy of standard chemotherapeutic agents. In this context, we have assessed the influence of an oxidative stress generated by the combination of ascorbate and the redox-active quinone menadione on the capacity of melphalan, a common alkylating agent, to induce apoptosis in a chronic myelogenous leukemia cell line. Our data show that oxidative stress did not inhibit but rather promoted cancer cell killing by melphalan. Interestingly, we observed that, in the presence of oxidative stress, the type of cell death shifted from a caspase-3 dependent apoptosis to necrosis because of an ATP depletion which prevented caspase activation. Taken together, these data suggest that ROS-generating agents could be useful in combination with standard chemotherapy, even if all the molecular consequences of such an addition remain to be determined.

1. Introduction

Because reactive oxygen species (ROS) are naturally produced by cells as a consequence of aerobic metabolism, cells have developed a sophisticated set of antioxidant molecules that prevents a toxic accumulation of these species. Interestingly, there is now a growing body of evidence supporting the existence of a redox dysregulation in cancer cells [1]. Compared with normal cells, malignant cells often exhibit increased levels of intracellular ROS [2], but also altered levels of antioxidant molecules [3,4]. The resulting endogenous oxidative stress favors tumor growth by promoting genetic instability [5,6], cell proliferation [7] and angiogenesis [8,9]. Several hypotheses have been formulated to explain the increased levels of ROS in cancer cells, such as mitochondrial dysfunction, aberrant metabolism or loss of p53 [10,11]. The expression of genes that are associated with tumor transformation, such as Ras, Bcr-Abl and c-Myc, were also found to induce the production of ROS, which actively participate in the tumorigenic functions of these proteins [6,12,13]. Taken together, these reports suggest that redox dysregulation is an "Achilles' heel" of tumors, and that such vulnerability can be exploited to selectively kill cancer cells. Indeed, because of their higher ROS generation, cancer cells are more dependent on the antioxidant systems and more vulnerable to further oxidative stress induced by pro-oxidant agents or compounds that inhibit the antioxidant systems (as reviewed by Wondrak [14]). For instance, increasing MnSOD protein by genetic manipulation or treating with a pharmacologic agent that mimics MnSOD (i.e. manganese porphyrin) results in an increased production of H_2O_2 that sensitizes lymphoma cells to dexamethasone-induced apoptosis [15,16]. Based on these observations, several ROS-generating agents are currently in clinical trials, either as single agents or in combination with standard therapy [1].

However, little is known about the potential consequences of the use of pro-oxidant drugs for the efficacy of standard chemotherapic agents, which could therefore decrease the interest of physicians for these approaches. In this context, we have studied the capacity of melphalan to induce apoptosis in chronic myelogenous leukemia cells (K562 cells), in the presence of an oxidative stress generated by the combination between ascorbate

Abbreviations: ROS, reactive oxygen species; NAC, N-acetyl-cysteine; CAT, catalase; LDH, lactate dehydrogenase; ANOVA, analysis of variance; PARP, poly(ADP-ribose) polymerase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside.

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and the redox-active quinone menadione (which will be abbreviated to A/M from now on), an oxidative stress-inducing system that has been extensively characterized by our laboratory [17–19]. In the absence of additional oxidative stress, melphalan induces a caspase-3 dependent apoptosis, as shown by multiple markers: increased DEVDase activity, cleavage of the poly(ADP-ribose) polymerase (PARP) protein and double staining Annexin-V/propidium iodide. However, in the presence of A/M, we observed a synergistic cytotoxic effect associated with a change in the type of cell death, from apoptosis to necrosis. Explaining this phenomenon, we observed that oxidative stress induced an ATP depletion that prevented caspase activation, although mitochondrial release of cytochrome c was not affected by the presence of ROS. Finally, using methylpyruvate, a cell-permeant metabolic substrate, or AICAR (aminoimidazole carboxamide ribonucleotide), a cell-permeant AMPK activator [20], we were able to replenish intracellular pools of ATP and to restore an apoptotic phenotype, thereby confirming that a decrease in ATP level is actually the molecular switch between necrosis and apoptosis in cancer cells exposed to an oxidant environment.

2. Materials and methods

2.1. Cell lines

The K562 cell line (chronic myeloid leukemia cells) was a gift of Dr. F. Brasseur (Ludwig Institute for Cancer Research-LICR-Brussels) and maintained in RPMI medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) from Gibco (Grand Island, NY, USA). The cultures were maintained at a density of 1–2 × 10^5 cells/ml and the medium was changed at 48–72 h intervals. All cultures were maintained at 37 °C in a 95% air and 5% CO_2 atmosphere at 100% humidity. In every experiment, K562 cells were incubated at a concentration of one million cells/ml. Inhibitors, melphalan and/or A/M were added directly into the incubation media at the indicated times.

2.2. Chemicals

Sodium ascorbate (vitamin C), menadione sodium metabisulfite (vitamin K3), catalase (CAT), methylpyruvate (MP), melphalan (Mel), N-acetylcysteine (NAC), sanguinarine, protease inhibitor cocktail and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Q-VD-OPh was purchased from R & D Systems (Minneapolis, MN, USA). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals (North York, ON, Canada). All other chemicals were ACS reagent grade.

2.3. Cell survival assays

Cell viability was estimated by measuring the activity of lactate dehydrogenase (LDH) according to the procedure of Wroblewski and Ladue [21] both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as 100 minus the ratio of released activity to the total activity.

2.4. Measurement of DEVDase activity

DEVDase activity, which reflects the caspase-3 activity, was monitored after 24 h of incubation. Briefly, after incubation with the test compounds, cells were washed twice with PBS, lysed and centrifuged. Cell supernatants were then incubated with a specific peptide substrate, Asp-Glu-Val-Asp-AFC (DEVD-AFC), from Enzo Life Sciences (Plymouth Meeting, PA, USA). Substrate cleavage was determined kinetically at room temperature using Victor X2 spectrophotometer (380 nm excitation, 500 nm emission) (Perkin Elmer, Waltham, MA, USA). Results are expressed as U/mg proteins, corresponding to the cleavage of 1 pmol of DEVD-AFC per min at 25 °C and at saturating substrate concentration, as originally described by Nicholson et al. [22]. The amount of proteins was determined by the method of Lowry using BSA as reference [23].

2.5. Measurement of ATP content

ATP content was determined by using the ATPlite assay from Perkin Elmer (Waltham, MA, USA) according to the procedures described by the supplier.

2.6. Annexin-V/propidium iodide staining

Cells were harvested at different times of incubation and stained with the Roche Annexin-V-FLUOS Staining kit (Mannheim, Germany) following manufacturer's instructions. Cells were then observed under a fluorescence microscope (Optika, Ponteranica, Italy), as previously described [24].

2.7. Immunoblotting

At the indicated times, cells were washed twice with ice-cold PBS and then resuspended in a lysis buffer containing 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), in 25 mM Tris–HCl pH 7.6 supplemented with Sigma Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA). The samples were kept on ice for 15 min before sonication (15 s, 100 W) and storage at −20 °C. The amount of protein was determined by the method of Bradford [25] using BSA as a reference and equal amounts of proteins (~30 μg) were subjected to SDS-PAGE (4–20% separating gels) followed by electroblot to nitrocellulose membranes. The membranes were blocked 1 h in TBS buffer (pH 7.4) containing 5% powdered milk protein followed by an overnight incubation with diluted antibodies in a fresh solution of powdered milk protein (1%, w/v) in TBS buffer. The membranes were washed and incubated for 60 min with a dilution of secondary antibody coupled to horseradish peroxidase. Anti-cyt c was a mouse monoclonal antibody diluted whereas anti-caspase-3 was a rabbit polyclonal antibody. They were used at 1:1000 and purchased respectively from BD Pharmingen (San Diego, CA) and Cell Signalling (Beverly, MA). Anti-b-actin was a mouse monoclonal antibody used at 1:10,000 and was purchased from Abcam (Cambridge, UK). Goat anti-rabbit antibody and rabbit anti-mouse polyclonal antibody were purchased respectively from Millipore (Billerica, MA) and DakoCytomation (Glostrup, Denmark).

2.8. Subcellular fractionation

Subcellular fractionation was performed as described previously [26]. Briefly, 5 × 10^6 cells were washed twice in phosphate-buffered saline and lysed by incubating for 30 s in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 350 mg/ml digitonin). Samples were then centrifuged at 14,000 × *g* for 2 min. Supernatants (cytosolic fraction) were collected in an equal volume of 2× SDS sample buffer containing 0.2% NP-40, 0.2% SDS, 1% sodium deoxycholate and 0.2% phenylmethanesulfonylfluoride in PBS pH 7.4. The pellets (mitochondria-rich fraction) were lysed by sonication in 1× SDS sample buffer. For both cytosolic and pellet fractions, proteins were quantified by the method of Bradord [25] and used for immunoblotting.

2.9. Statistical analysis

Results are presented as mean values and the error bars represent the standard error of the mean. Data were analyzed with

GraphPad Prism software (GraphPad Software, San Diego, CA, USA), using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's test for significant differences between means. For statistical comparison of results at a given time point, data were analyzed using Student's *t* test.

3. Results

3.1. Influence of oxidative stress on melphalan-induced toxicity

We first compared the sensitivity of K562 cells towards the cytotoxicity of either melphalan or an oxidative stress generated by combining ascorbate and menadione (A/M). The latter system is initiated by electron transfer from ascorbate ($AscH^-$) to quinone (Q), as illustrated in Eq. (1):

$$AscH^- + Q \rightarrow SQ^{\bullet -} + Asc^{\bullet -} + H^+ \quad (1)$$

$$SQ^{\bullet -} + O_2 \rightarrow Q + O_2^{\bullet -} \quad (2)$$

Eq. (2) shows the rapid reoxidation of the semiquinone free radical ($SQ^{\cdot -}$) to its quinone form (Q) by molecular oxygen leading to the formation of ROS derived from superoxide anion ($O_2^{\cdot -}$), such as hydrogen peroxide (H_2O_2) or hydroxyl radicals ($HO^{\cdot}$). The incubation of K562 cells in the presence of increasing amounts of melphalan results in a progressive leakage of lactate dehydrogenase (LDH) into the incubation medium, particularly evident at 48 h, reflecting the occurrence of cell death (Fig. 1A). On the other hand, the viability of K562 cells exposed to the A/M combination is more quickly compromised. Indeed, toxicity was detected as early as 24 h after the exposure, and slightly increased at 48 h (Fig. 1B). It is worth noting that, in the experiments using A/M, we always use the same concentration of ascorbate (2 mM) but varying amounts of menadione (from 2 to 10 μM). The explanation to this atypical experimental design relies on the particular role played by menadione in reactions (1) and (2), which can be compared to a role of catalyst for ascorbate oxidation. Therefore, modulating the concentrations of menadione alone represents a convenient mean to progressively increase the oxidative stress generated by this system, as described in our previous reports [27]. The use of Q-VD-Oph, a broad spectrum caspase inhibitor, completely prevented cell death induced by melpahalan (Fig. 1C). However, Q-VD-Oph had no effect on A/M toxicity (Fig. 1D). These results suggest that melphalan, but not A/M, triggers a caspase-dependent apoptosis in K562 cells.

We then assessed the toxicity of combining melphalan with A/M. As shown in Fig. 2A, combining low doses of melphalan (up to 50 μM) with mild oxidative injuries by A/M (2 mM of ascorbate and 2 or 5 μM of menadione) results in an increased toxicity compared to each agent used by themselves. At higher dosage of melphalan (i.e. 100 μM), this apparent synergistic effect was lost and a rather additive toxicity appeared. To understand the part due to oxidative stress in the increased cell killing observed in the presence of the two treatments, we used the hydrogen peroxide (H_2O_2)-detoxifying enzyme catalase. As shown in Fig. 2B, the

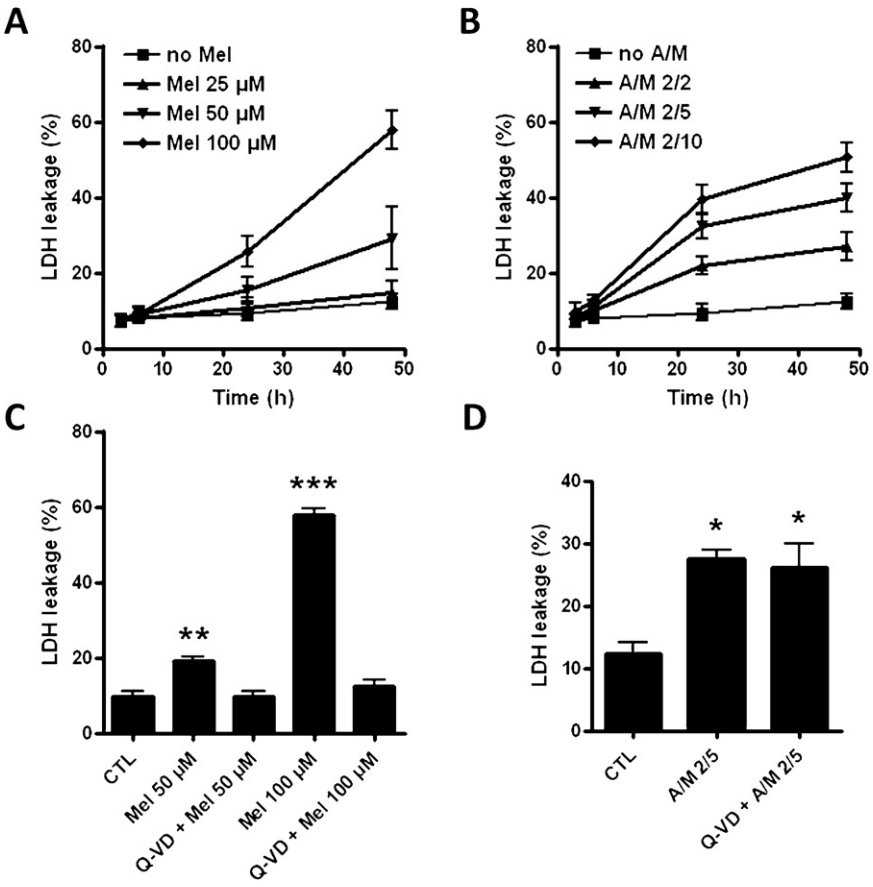

Fig. 1. Respective toxicities of melphalan and A/M on K562 cells. K562 cells were incubated with increasing concentrations of melphalan (A) or with a combination between 2 mM ascorbate and varying concentrations of menadione (from 2 to 10 μM) (B). Cell death was assessed at different time points by measuring LDH leakage, as described under Section 2. Results are means of three separate experiments. (C) Cells were incubated with 50 or 100 μM of melphalan, either in the absence or the presence of 10 μM of Q-VD-Oph. Cell death was assessed at 48 h after the exposure to melphalan, by measuring LDH leakage. Results are means of four separate experiments. (D) Cells were incubated with 2 mM ascorbate and 5 μM menadione, either in the absence or in the presence of 10 μM of Q-VD-Oph. Cell death was measured as in (C). Results are means of three separate experiments. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

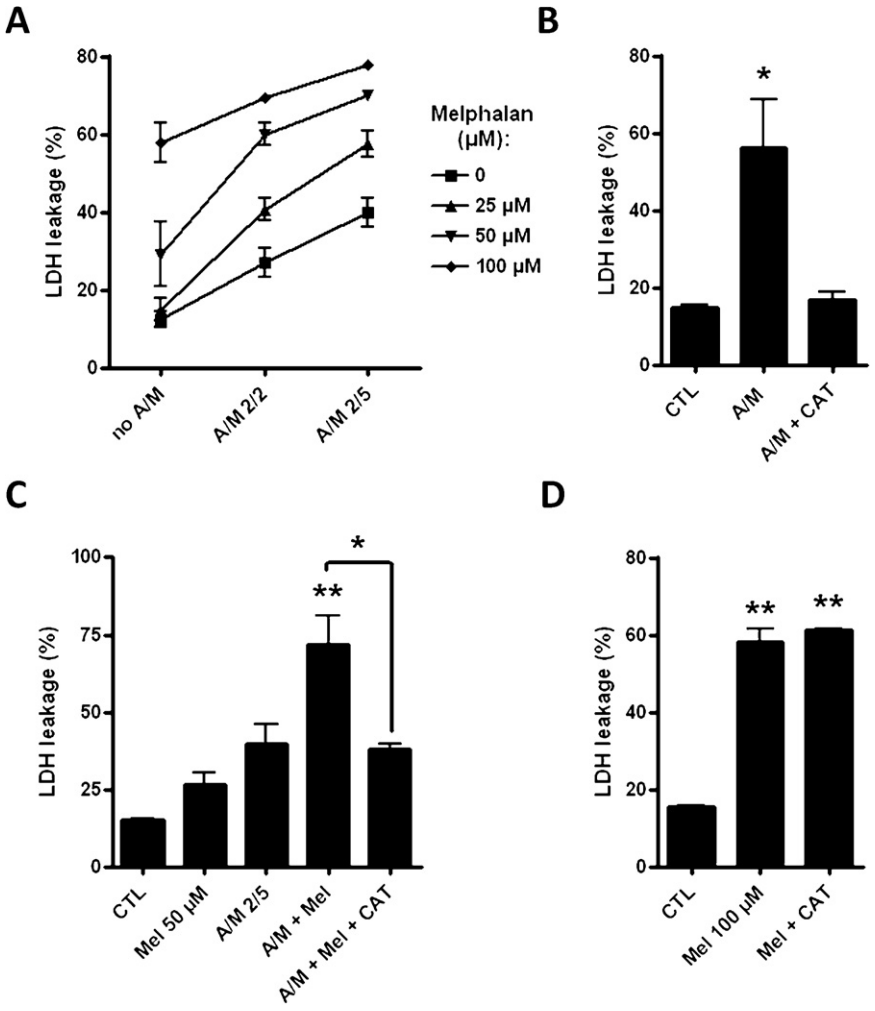

Fig. 2. Synergistic cancer cell killing by combining A/M and melphalan. (A) K562 cells were incubated with increasing concentrations of melphalan either in the absence or the presence of the A/M combination used at 2 mM ascorbate and 2 or 5 μM of menadione. Cell death was measured at 48 h by measuring LDH leakage, as described under Section 2. (B) Cells were incubated with 2 mM ascorbate and 10 μM menadione, in the presence or the absence of the antioxidant enzyme catalase (CAT), used at 100 U/ml. Cell death was measured at 48 h by measuring LDH leakage, as previously described. (C) Cells were incubated with 50 μM melphalan, in the presence or the absence of the A/M combination (used at 2 mM ascorbate and 5 μM menadione) and catalase (100 U/ml). Cell death was measured at 48 h, as in (B). (D) Cells were incubated with 100 μM melphalan, in the presence or the absence of catalase (100 U/ml). Cell death was measured at 48 hours, as in (B). Results presented in (A), (B), (C) and (D) are means of three separate experiments. (*) $p < 0.05$, (**) $p < 0.01$.

addition of catalase into the incubation medium completely suppressed the toxic effects of 2 mM of ascorbate and 10 μM of menadione, in agreement with our previous reports demonstrating the critical role played by H_2O_2 in A/M toxicity [26,27]. Indeed, the redox-cycling that occurs between ascorbate and menadione generates extracellular H_2O_2, which can diffuse into cancer cells and provoke deleterious effects. In the presence of catalase, the overall toxicity of combined melphalan and A/M treatments was considerably decreased, suggesting that oxidative stress itself is the most important factor that determines the synergistic effect observed when combining A/M and melphalan (Fig. 2C). The remaining toxicity observed in these specific conditions is likely due to the pro-apoptotic properties of melphalan. Indeed, the activity of this compound was not affected by the presence of catalase (Fig. 2D).

3.2. Characterization of the type of cell death

As previously suggested by our first set of experiments, we observed that cells treated with melphalan (used at 100 μM) for 24 h exhibited an apoptotic phenotype, as shown by Annexin-V staining (Fig. 3A). These data also indicate that the release of LDH that we observed at 48 h is likely the result of secondary necrosis [28]. Interestingly, when melphalan was combined to A/M, apoptosis (Annexin-V positive, PI negative cells) was no longer observed and most cells presented a necrotic profile (positive for both Annexin-V and PI). This observation was in agreement with further data showing that Q-VD-Oph did not offer any protection when cells were treated by both melphalan and A/M (Fig. 3B). The activity of caspase-3 was also assessed, by measuring the DEVDase activity in cell lysates. When used as a single agent, melphalan induced a strong DEVDase activity, suggesting that melphalan-induced apoptosis is likely caspase-3 dependent (Fig. 3C). However, in the presence of the oxidative stress driven by A/M, DEVDase activity was considerably decreased, being not statistically different from the activity observed in control lysates. These data suggest that the addition of A/M to melphalan provokes a shift in the type of cell death, from a caspase-3 dependent apoptosis to a necrotic type of cell death.

From the literature, it is known that a lack of caspase-3 activity can occur in certain circumstances. One is the inhibition of caspase-3 processing because of a failure in ATP generation [29,30]. Explaining this phenomenon, it has been demonstrated that the presence of ATP is absolutely required for the assembly of the multimeric protein complex named the 'apoptosome', which

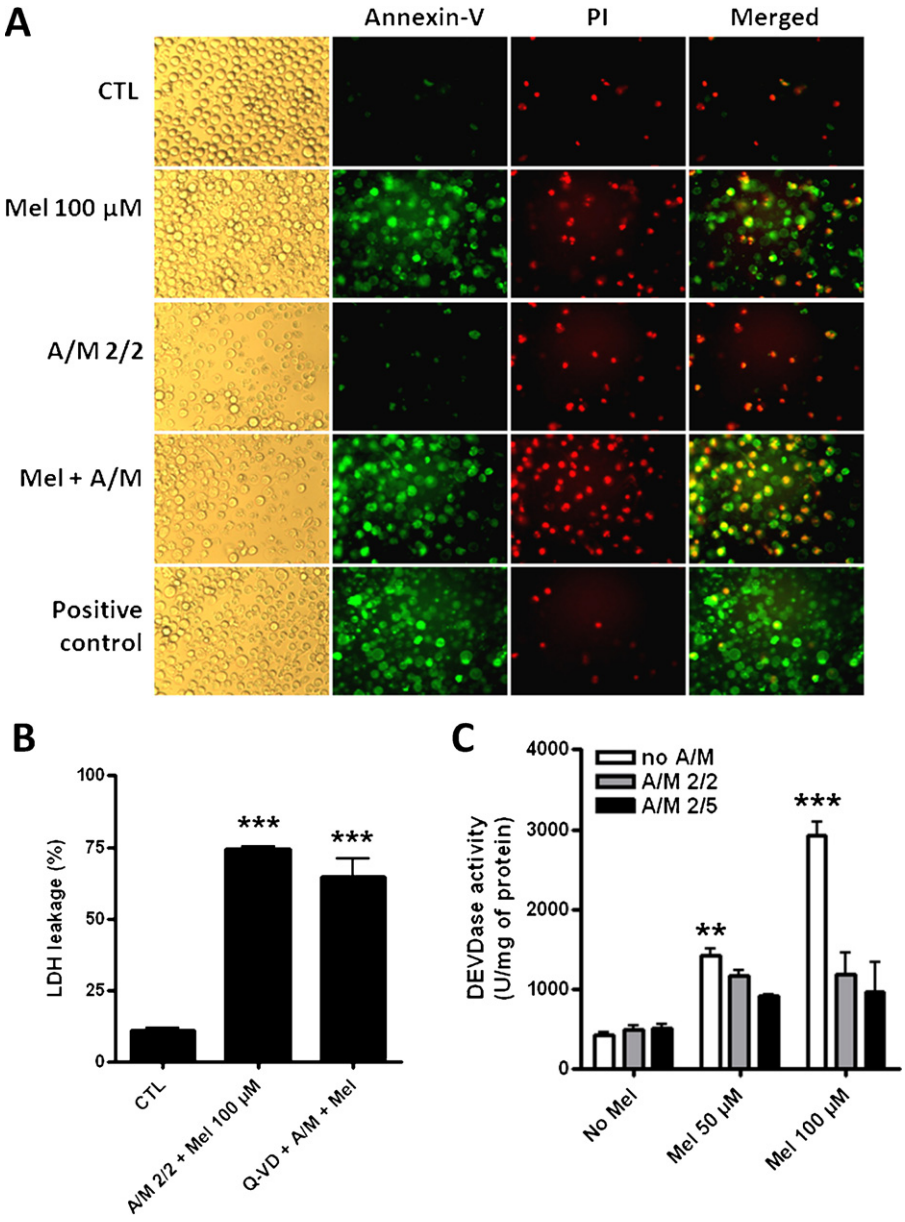

Fig. 3. The presence of A/M induces a switch in the type of cell death. (A) K562 cells were treated with 100 μM melphalan, in the presence or the absence of the A/M combination (used at 2 mM ascorbate and 2 μM menadione). Twenty-four hours after the exposure to the toxic agents, a double staining with annexin-V/propidium iodide was performed as described under Section 2. Sanguinarine (10 μM) was used as a positive control for apoptosis, as previously described [26]. (B) Cells were incubated with 100 μM of melphalan and A/M combination (used at 2 mM ascorbate and 2 μM menadione), either in the absence or the presence of 10 μM of Q-VD-Oph. Cell death was measured at 48 h by measuring LDH leakage, as previously described. Results are means of three separate experiments. (C) Cells were incubated with two concentrations of melphalan (50 and 100 μM), either in the absence or the presence of the A/M combination (used at 2 mM ascorbate and 2 or 5 μM of menadione). Aliquots of cell suspension were taken after 16 h of incubation and DEVDase activity (which reflects caspase-3 activity) was measured as described under Section 2. Results are means of three separate experiments. (**) $p < 0.01$, (***) $p < 0.001$.

drives caspase activation in the mitochondrial (or intrinsic) pathway of apoptosis [31]. Another cause of absence of caspase-3 activity despite the presence of apoptotic inducers is the direct inhibition of its enzymatic activity because of oxidative modifications of a critical cysteine residue located in the active motif [32,33]. In order to discriminate between these two possibilities, we assessed different steps of caspase activation: procaspase-3 processing and cytochrome c leakage. As shown in Fig. 4A, the exposure to melphalan results in procaspase-3 processing, as evidenced by the presence of caspase-3 active subunits of 19 and 17 kDa. Treatment by A/M did not lead to procaspase-3 processing and, when coupled to melphalan, A/M strongly repressed its activation. This suggests that caspase-3 inhibition observed in the presence of A/M does not result from oxidative modifications of the active form of the protein, but is rather due to the blockade of an ATP-dependent step, upstream of caspase-3 activation. Moreover, cell fractionation experiments showed that cytochrome c release from mitochondria was constantly induced by melphalan exposure, regardless of the presence or absence of oxidative stress generated by A/M (Fig. 4B). Taken together, these results suggest that the molecular switch responsible for the change in the type of cell death observed in the presence of oxidative stress generated by A/M affects a step located downstream of cytochrome c release but upstream of caspase-3 activation. Since we had previously observed that A/M treatment of cancer cells provokes a decrease of the intracellular ATP levels [19,34], we then sought to determine

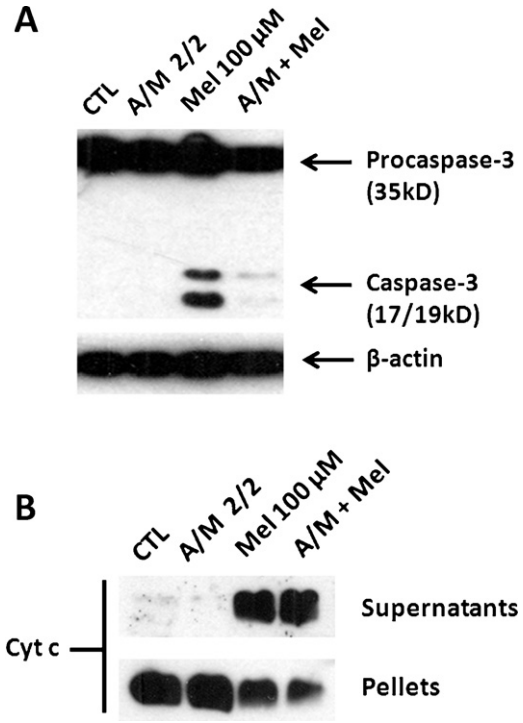

Fig. 4. Co-incubation with A/M prevents caspase-3 activation but not melphalan-induced cytochrome c release from mitochondria. (A) K562 cells were treated with 100 μM melphalan, in the presence or the absence of the A/M combination (used at 2 mM ascorbate and 2 μM menadione). After 16 h of incubation, cells were washed twice with PBS, lyzed in RIPA buffer and were subjected to immunoblotting, as described under Section 2. (B) Cells were incubated in the same conditions as described in (A). After 16 h of incubation, cells were washed twice with PBS and subcellular fractionation was performed as under Section 2. Supernatants represent the cytosolic fractions whereas the pellets are the mitochondria-rich fractions.

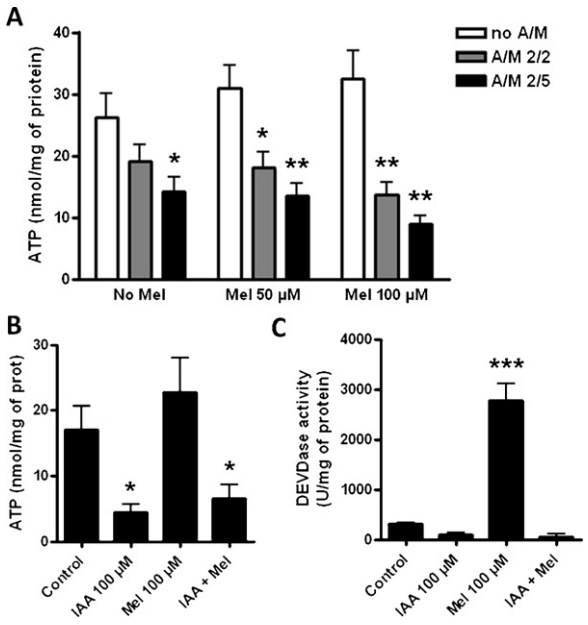

Fig. 5. ATP depletion is responsible for the inhibition of caspase-3 activity. (A) Cells were incubated with two concentrations of melphalan (50 and 100 μM), either in the absence or the presence of the A/M combination (used at 2 mM ascorbate and 2 or 5 μM of menadione). Intracellular ATP levels were determined after 6 h of incubation, as described under Section 2. Results are means of four separate experiments. (B) Cells were incubated with 100 μM melphalan, either in the presence or the absence of 100 μM iodoacetate. Intracellular ATP levels were determined after 6 hours of incubation, as in (A). Results are means of three separate experiments. (C) Cells were incubated as in (A) and aliquots of cell suspension were taken after 16 h of incubation. DEVDase activity was measured as described under Section 2. Results are means of three separate experiments. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

the putative influence of A/M on ATP in this model, and to evaluate the consequences for cell death. Indeed, we have previously published that the oxidative stress generated by A/M leads to the occurrence of DNA damage which can provoke a strong activation of the Poly(ADP-ribose) polymerase enzyme (PARP) [19]. Excessive PARP activity consumes NAD^+, and because NAD^+ is required for glycolysis, it renders cells unable to use glucose as a metabolic substrate. Given that cancer cells primarily rely on glycolysis for their energetic supply, the glycolysis arrest provoked by A/M can thus cause a rapid depletion of ATP.

3.3. ATP levels as the molecular switch between apoptosis and necrosis

Measurements of ATP in K562 cells revealed that a 6-h exposure to melphalan did not change the energetic status of cancer cells (Fig. 5A). However, A/M treatment alone induced thirty to forty-five percent decrease in ATP levels depending whether A/M was used with 2 or 5 μM of menadione. In the presence of both melphalan and A/M, a thirty to seventy percent decrease in ATP levels was observed.

To study the impact of an ATP decrease on the capacity of melphalan to induce apoptosis, we sought to inhibit ATP production in K562 cells using iodoacetic acid (IAA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase. We decided to use a glycolysis inhibitor because cancer cells present an altered energetic metabolism. Indeed, they primarily rely on glycolysis for energy production, even in the presence of oxygen, a phenomenon described as "aerobic glycolysis" or "Warburg effect" [35]. The incubation of cells in the presence of 100 μM of IAA led to a strong decrease of intracellular ATP (approximately of 70%), either in the absence or in the presence of melphalan (Fig. 5B). IAA also suppressed melphalan-driven DEVDase activity (Fig. 5C), thereby providing a proof-of-concept that ATP depletion is able to prevent caspase-activation.

To confirm the role played by intracellular ATP concentration as a molecular switch between apoptosis and necrosis in our model of oxidative-stress exposed cancer cells, we attempted to restore ATP levels using methylpyruvate (MP), a cell-permeant pyruvate derivative which is preferentially metabolized in mitochondria to sustain ATP production [36]. As evidenced by a double Annexin-V/PI staining, incubation in the presence of methylpyruvate prevents the occurrence of necrosis in cells treated by both melphalan and A/M, in a similar manner to that of catalase (Fig. 6A). Intracellular ATP measurements further confirmed that methylpyruvate restored ATP levels in K562 cells treated by both A/M and melphalan (Fig. 6B), and restored DEVDase activity in the same condition (Fig. 6C). Interestingly, similar results were reproduced with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a cell-permeant AMPK activator. AICAR is readily taken up by cells and is then phosphorylated by adenosine kinase to form ZMP, an AMP analog, which activates AMPK [20]. By switching off ATP-consuming pathways and turning-on alternative pathways for ATP generation (e.g. fatty acid oxidation), AMPK activation induces profound alterations in cell metabolism that favor ATP production and protect cells against various stresses [37,38]. In our hands, AICAR was able to prevent the ATP depletion induced by A/M and restored caspase-3 activity in cells treated by both A/M and melphalan (Fig. 7A and B). Taken together, the effects of methylpyruvate and AICAR confirm that the switch in the type of cell death that we observed in the presence of A/M is due to the ATP depletion provoked by oxidative stress.

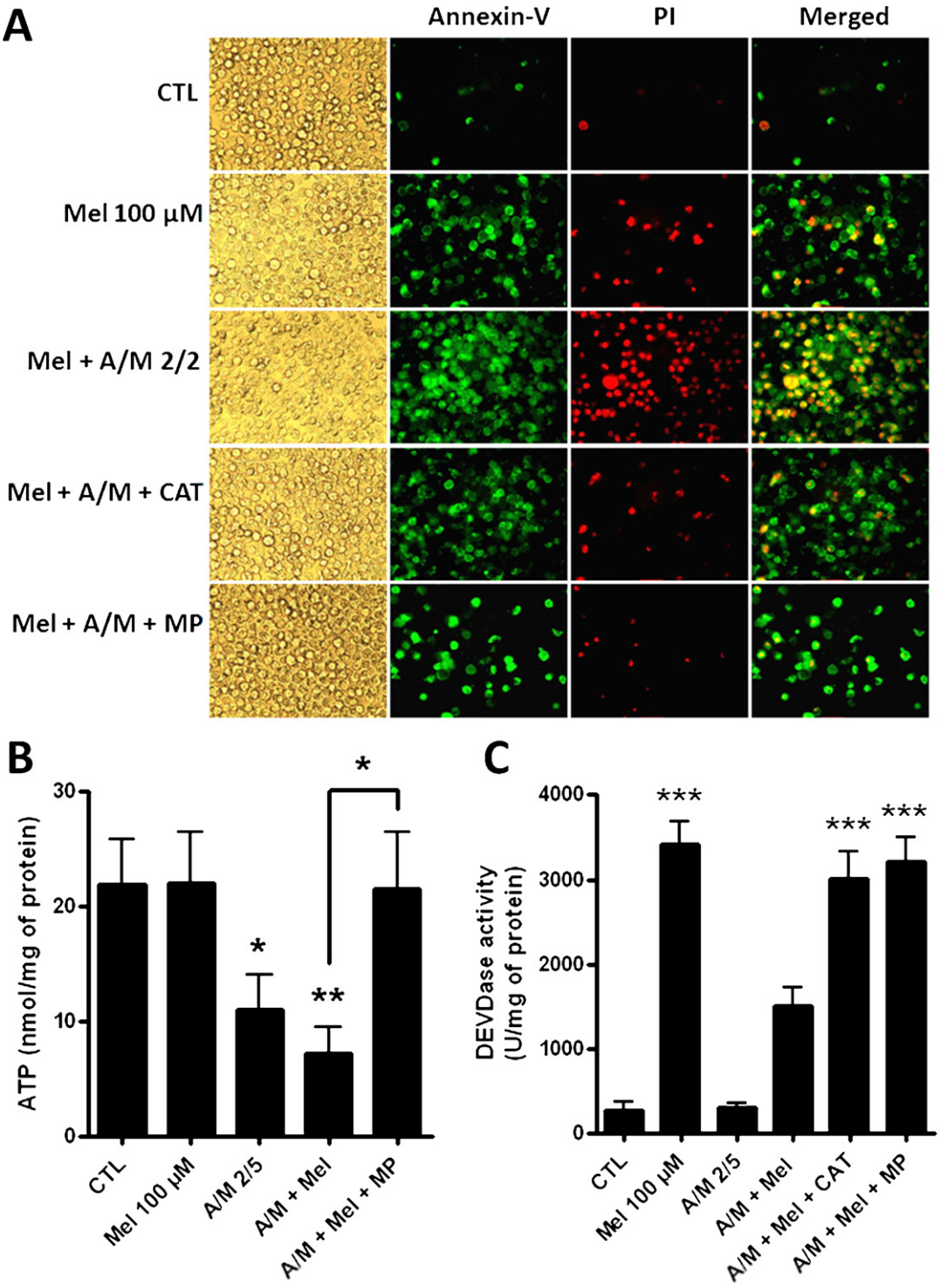

Fig. 6. Methylpyruvate restores ATP levels in A/M-treated cells and reverses the switch in the type of cell death. (A) K562 cells were treated with 100 μM melphalan and or the A/M combination (used at 2 mM ascorbate and 2 μM menadione), in the presence or the absence of catalase (100 U/ml) or methylpyruvate (MP, used at 5 mM). Twenty-four hours after the exposure to these compounds, a double staining with annexin-V/propidium iodide was performed as described under Section 2. (B) Cells were incubated with 100 μM melphalan, either in the presence or the absence of the A/M combination (used at 2 mM ascorbate and 5 μM menadione) and 5 mM MP. Intracellular ATP levels were determined after 6 h of incubation, as described under Section 2. Results are means of seven separate experiments. (C) Cells were incubated as in (B) and aliquots of cell suspension were taken after 16 h of incubation. DEVDase activity was measured as described under Section 2. Results are means of three separate experiments. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

4. Discussion

Many agents that induce cancer cell death through redox mechanisms are currently under preclinical and/or clinical evaluation (for an extensive review of these trials, the readers are referred to Wondrak [14]). Based on their mechanism of action, these compounds can be separated into two categories: the ones that generate ROS, and the ones that inhibit the antioxidant systems. Redox cyclers such as anthracyclines (e.g. doxorubicin) and naphtoquinones (e.g. menadione) are well-known to generate superoxide anion and belong to the first category. Isothiocyanate derivatives such as benzylisothiocyanate (BITC), phenylethyli-

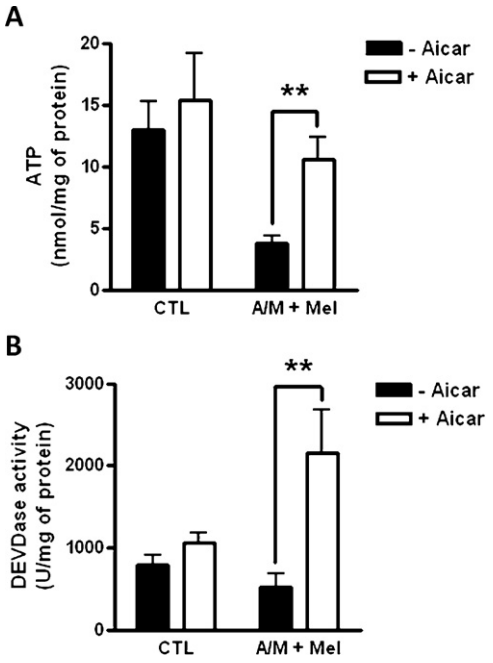

Fig. 7. The AMPK activator AICAR confirms the critical role of ATP as the molecular switch between apoptosis and necrosis in cells treated by both melphalan and A/M. (A) K562 Cells were pre-incubated with 300 μM of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), for 24 h. Then, they were exposed to 100 μM melphalan, either in the presence or the absence of the A/M combination (used at 2 mM ascorbate and 5 μM menadione). Intracellular ATP levels were determined after 6 h of incubation, as described under Section 2. Results are means of five separate experiments. (B) Cells were incubated as in (A) and aliquots of cell suspension were taken after 16 h of incubation. DEVDase activity was measured as described under Section 2. Results are means of four separate experiments. (**) $p < 0.01$.

sothiocyanate (PEITC), and sulphoraphane provide good examples of compounds from the second category. These molecules rapidly conjugate with glutathione (GSH) causing a depletion of the GSH pool [39]. This has dramatic consequences for cancer cells that frequently present increased levels of intrinsic oxidative stress and therefore rely on GSH for redox adaptation [1]. It is worth noting that the effects of some molecules can sometimes be explained by multiple mechanisms. This is the case for arsenic trioxide (As_2O_3), which increases superoxide production by impairing mitochondrial respiration [40], and also inhibits the thioredoxin antioxidant system [41].

Although the use of these redox-based agents offers a promising new way of treating cancer, a major question remains to determine whether these compounds should be used as combinatorial or stand-alone drugs. Given that cancer cells generally present a dysregulation of multiple pathways, current chemotherapy regimens often associate different molecules, each targeting a specific pathway. In this context, an important goal of preclinical trials should be to characterize the influence of redox-based chemotherapeutics on the efficacy of standard chemotherapeutic agents.

Here, we have used a combination of ascorbate and menadione as a model of pro-oxidant strategy and we determined its effects on melphalan, a standard alkylating agent. The A/M treatment was used for two different reasons: (1) this combination generates a constant oxidative stress that can be fine-tuned by varying the concentration of menadione [27]; (2) this treatment has been granted by the FDA for the treatment of metastatic or locally advanced, inoperable transitional cell carcinoma of the urothelium (stages III and IV bladder cancer) and the results of a first clinical trial using an oral combination of A/M (Apatone©) have been published [42]. It should be noted that other systems involving the combination of ascorbate and a catalyst have been described in the literature. For instance, the concomitant use of ascorbate and manganese porphyrins produces pro-oxidative effect that also kills cancer cells. Indeed, in the presence of ascorbate, Mn porphyrins are reduced, bind oxygen and reduce it to peroxide, leading to oxidative stress [43,44]. Interestingly, the existence of a similar interaction between ascorbate and unknown metalloproteins has also been postulated as an explanation for the pro-oxidant and antitumoral effects of pharmacologic doses of ascorbate [45–47].

Our data show that A/M addition to melphalan results in increased cancer cell killing as compared to either A/M or melphalan alone (Fig. 2A). However, this increased anticancer activity is accompanied by a switch in the type of cell death. Indeed, melphalan alone clearly induced a caspase-3 dependent apoptosis in K562 cells, as shown by Annexin-V staining (Figs. 3A and 6A), DEVDase activity (Fig. 3C), the inhibitory effect of a broad caspase inhibitor (Fig. 1C), cytochrome c leakage and procaspase-3 processing (Fig. 4A and B). These results are in agreement with previous reports showing that melphalan induces apoptosis in cancer cells through Bax relocalization, cytochrome c leakage and caspase activation [48,49]. When coupled to A/M, however, the resulting cell death was rather necrotic. Explaining this phenomenon, we observed that the addition of A/M (even under conditions of mild oxidative stress, i.e. using 2 μM of menadione) prevents procaspase-3 processing in K562 cells exposed to melphalan. Since cytochrome c leakage is not inhibited by the presence of A/M (Fig. 4B), it is tempting to suggest that the main consequence of ATP depletion is the inhibition of apoptosome formation. Indeed, the conformational changes of Apaf-1 needed for apoptosome-mediated caspase activation require the binding of dATP/ATP after Apaf-1 interaction with cytochrome c [31,50]. Moreover, it has previously been demonstrated by Leist et al. that a 50% decrease of intracellular ATP concentration is sufficient to block the progression of apoptosis. This suggests that apoptosis and necrosis are the two extremes of a continuum of possible types of cell demise, whose shape would be decided by the availability of ATP [29]. The critical role played by ATP in deciding the cellular outcome in our model is supported by several lines of evidence. First, glycolysis inhibition by iodoacetate led to a decrease of intracellular ATP and the inhibition of DEVDase activity in melphalan-treated cells. Second, the use of a cell-permeant pyruvate derivative replenished ATP levels in cells treated by both A/M and melphalan, thereby allowing apoptosome assembly, caspase-3 activation and restoring an apoptotic phenotype. Third, similar results were observed with an AMPK activator, namely AICAR [20].

Taken together, these observations do not diminish the interest for the A/M combination in cancer therapy. Indeed, A/M induced an overall increase in cancer cell killing in the presence of melphalan, suggesting that its use as a combinatorial drug might be beneficial. However, several questions are still open, notably about the occurrence of these processes in vivo, as well as the question to know whether other pro-oxidant strategies can induce a similar switch in the type of cell death when used in combination with melphalan or other chemotherapeutic drugs.

Beyond the particular model presented in this study, our data illustrate that combining novel pro-oxidant strategies to standard chemotherapeutic regimens can have unexpected consequences. As for every novel drug candidate, novel pro-oxidant strategies need appropriate pre-clinical trials, even if some agents used in these approaches are generally considered as low-toxic (e.g. ascorbate). This is the only way to avoid mixed results and controversy.

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