UICC
global cancer control

IJC
International Journal of Cancer

The DNA methyltransferase inhibitors zebularine and decitabine induce mitochondria-mediated apoptosis and DNA damage in p53 mutant leukemic T cells

María J. Ruiz-Magaña[1], Jose M. Rodríguez-Vargas[2], Jorge C. Morales[1], Manuel A. Saldivia[1], Klaus Schulze-Osthoff[3] and Carmen Ruiz-Ruiz[1]

[1] Unidad de Inmunología, IBIMER, Universidad de Granada, Granada, Spain
[2] Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Granada, Spain
[3] Interfaculty Institute of Biochemistry, University of Tübingen, Tübingen, Germany

DNA methyltransferase (DNMT)-inhibiting nucleoside analogs reactivate the expression of tumor suppressor genes and apoptosis-related genes silenced by methylation, thus favoring the induction of apoptosis in tumor cells. Moreover, induction of DNA damage seems to contribute to their antitumor effect. However, the apoptotic signaling pathway induced by these demethylating drugs is not well understood. Here, we have investigated the induction of apoptosis by two nucleoside DNMT inhibitors, decitabine and zebularine, in leukemic T cells. Both inhibitors induced caspase-dependent apoptosis in Jurkat, CEM-6 and MOLT-4 leukemia T cell lines, all with mutant p53, whereas resting and activated normal T lymphocytes were highly resistant to these demethylating agents. Although decitabine and zebularine showed different ability to induce apoptosis and cell cycle arrest among the three cell lines, they similarly activated the intrinsic apoptotic pathway by inducing mitochondrial alterations such as Bak activation, loss of transmembrane potential and generation of reactive oxygen species (ROS). Accordingly, Bcl-2- and Bcl-x_L-overexpressing Jurkat cells, as well as caspase-9-deficient Jurkat cells, were resistant to apoptosis induced by decitabine and zebularine. Interestingly, ROS production seemed to be necessary for the induction of apoptosis. Apoptotic events, such as Bak and caspase activation, started as soon as 20 hr after treatment with either decitabine or zebularine. In addition, progression of apoptosis triggered by both DNMT inhibitors was paralleled by the induction of DNA damage. Our results suggest that the mitochondrial apoptotic pathway activated by decitabine and zebularine in p53 mutant leukemic T cells depends mainly on the induction of DNA damage.

Abnormal DNA methylation of CpG sequences is one of the most common epigenetic modifications observed in tumors. Hypermethylation of promoter regions of tumor suppressor genes causes their silencing, thus favoring tumor progression.[1–3] This has focused attention on drugs that reactivate methylated cancer genes as a promising antitumor strategy.

The cytosine nucleoside analogs 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine) are the best known demethylating agents.[4] These compounds are metabolized and phosphorylated inside the cell and then incorporated into DNA, where they are recognized by DNA methyltransferases (DNMTs), the enzymes responsible for DNA methylation. The covalent complexes formed by the abnormal bases and DNMT result in degradation of the trapped enzymes.[5] Both DNMT inhibitors have been approved by the Food and Drug Administration for the treatment of myelodysplastic syndromes (MDS), despite being quite toxic and unstable.[5,6] Another cytidine analog, zebularine, although originally described as a cytidine deaminase inhibitor, also has DNA demethylating activity. *In vitro* and *in vivo* studies have demonstrated that zebularine is highly stable and shows minimal toxicity.[7,8] It exhibits selective activity against various cancer cells when compared to normal fibroblasts.[9]

Tumor cell exposure to demethylating agents causes the upregulation of cell cycle-associated genes, such as p14, p15 or p16, and genes related to the induction of apoptosis, such as caspase-8 or Apaf-1, that are silenced by hypermethylation.[3,10–13] As a consequence, demethylating agents induce cell cycle arrest and apoptosis in tumor cells and sensitize them to other chemotherapeutic drugs.[12,13] On the other hand, nucleoside DNMT inhibitors are able to form complexes with DNA-DNMT that induce DNA damage and cytotoxicity,[14,15] which suggests that these DNMT inhibitors may exert their antitumoral effects by both mechanisms, dependent and independent of their demethylating action.

Key words: decitabine, zebularine, leukemia, apoptosis
Conflict of interest: None
Grant sponsor: Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo; **Grant number:** PI060712
DOI: 10.1002/ijc.26107
History: Received 23 Sep 2010; Accepted 15 Mar 2011; Online 31 Mar 2011
Correspondence to: Carmen Ruiz-Ruiz, IBIMER, Centro de Investigación Biomédica, Avda. del Conocimiento s/n, 18100 Armilla, Granada, Spain, Tel.: +34-958-241000, ext. 20025, Fax: +34-958-249015, E-mail: mcarmenr@ugr.es

Several reports have discussed the contribution of these two mechanisms of action, hypomethylation and DNA damage, the involvement of the tumor suppressor protein p53 and the role of the mitochondria in the induction of apoptosis by decitabine, particularly in myeloid leukemias.[15–18] In contrast, much less is known about the signals involved in zebularine-induced apoptosis.

Aberrant DNA methylation has been reported to be associated with gene silencing in T-cell acute lymphoblastic leukemia.[19–21] Moreover, the methylation status of several genes seems to correlate with the tumor response to treatment and the outcome of patients,[19,22] which suggest the therapeutic potential of DNMT inhibitors in this hematologic malignancy. Nevertheless, the cytotoxic effect of demethylating agents in leukemic T cells has not been described in detail. The aim of our study was to compare and characterize the induction of apoptosis by zebularine and decitabine in leukemic and normal T cells. Our results show that both DNMT inhibitors similarly activated the intrinsic apoptotic pathway in leukemic T cells in a p53-independent manner, at doses that, especially in the case of zebularine, were nontoxic for normal T lymphocytes. In addition, apoptosis induced by both inhibitors occurred simultaneously to the induction of DNA damage. However, differences were found in the ability of decitabine and zebularine to induce cell cycle arrest as well as in the sensitivity of leukemic T cell lines to both demethylating agents.

Material and Method

Reagents and antibodies

5-Aza-2′-deoxycytidine (decitabine), dihydroethidium (DHE), phytohemagglutinin, mouse anti-β-actin antibody and monoclonal anti-p53 antibody were from Sigma-Aldrich (St. Louis, MO). 1-(β-D-Ribofuranosyl)-1,2-dihydropyrimidin-2-one, 2-pyrimidone-1-β-D-riboside (zebularine), anti-Bak (Ab-1) monoclonal antibody and anti-Chk1 (pSer^{317}) rabbit polyclonal antibody were obtained from Calbiochem (Darmstadt, Germany). Z-VAD-FMK, a wide-spectrum caspase inhibitor, was from Bachem (Bubendorf, Switzerland). Mouse anti-human CD28 was from eBioscience (San Diego, CA). Anti-human caspase-8 monoclonal antibody was purchased from Cell Diagnostica (Munster, Germany). Anti-human caspase-9 monoclonal antibody was from R&D Systems (Minneapolis, MN). Mouse anti-PARP was from BD Biosciences (San Jose, CA). Monoclonal antibody anti-Bcl-2 was from Dako (Glostrup, Denmark). Mouse monoclonal antibody against Bcl-x_L and monoclonal antibody anti-histone H1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal DNMT1 antibody was from Abcam (Cambridge, UK). Anti-phospho-histone H2AX (Ser139) monoclonal antibody was from Upstate/Millipore (Billerica, MA). Rabbit polyclonal antibody phospho-p95/Nbs1 (Ser343) was obtained from Cell Signaling Technology (Danver, MA). The fluorescent cationic lipophilic dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was from Molecular Probes (Carlsbad, CA). Manganese-porphyrin Mn(III)TMPyP was obtained from Cayman Chemical (Ann Arbor, MI).

Cells and cell culture

Blood samples were obtained from healthy donors by informed consent and collected into citrates tubes. Peripheral blood T lymphocytes were then isolated and activated as previously described.[23] The human leukemic T cell lines Jurkat, CEM-6 and MOLT-4 were kindly provided by Dr. Abelardo López-Rivas (CABIMER, Sevilla, Spain). Jurkat cells deficient in caspase-9 and caspase-9-reconstituted Jurkat cells have been previously published.[24] Cell lines were all maintained in culture in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1 mM L-glutamine, penicillin and streptomycin at 37°C in a humidified 5% CO_2, 95% air incubator. Jurkat cells stably overexpressing Bcl-2 and Bcl-x_L were generously provided by Dr. Jacint Boix (Departamento de Ciencias Médicas Básicas, Universidad de Lleida, Spain) and maintained in culture medium with 1 mg/ml G418 sulfate (Sigma Chemical).

Cell cycle and apoptosis analyses

Cell cycle profiles and hypodiploid apoptotic cells were detected by flow cytometry according to the published procedures.[25] Briefly, cells were washed with PBS, fixed in cold 70% ethanol and then stained with propidium iodide while treating with RNase. Cellular DNA content was measured in a FACScan cytometer with the Cell Quest software (BD Biosciences) and using logarithmic amplification for quantification of sub-G1 apoptotic cells and linear amplification for quantification of cells arrested in the G2/M phase.

Determination of mitochondrial membrane potential and ROS generation

Mitochondrial membrane potential ($\Delta\psi_m$) and reactive oxygen species (ROS) production were analyzed by flow cytometry. Briefly, cells (2.5×10^5) were incubated in RPMI 1640 medium supplemented with 5% FBS and containing the oxidant-sensitive probe DHE (2 μM) and the fluorescent potentiometric dye $DiOC_6(3)$ (10 nM), for 20 min at 37°C in the dark, followed by quantitative analysis in a FACScan cytometer with the Cell Quest software.

Flow cytometry analysis of Bak activation

For detection of activated Bak, cells (2.5×10^5) were washed in PBS, fixed in 0.25% paraformaldehyde for 5 min on ice and washed three times with PBS. Cells were then incubated with 1:50 anti-Bak antibody in staining buffer (PBS containing 1% FBS and 100 μg/ml digitonin) for 30 min at room temperature. After washing, cells were incubated in staining buffer with 0.2 μg Alexa Fluor 488-labeled goat anti-mouse (Molecular Probes) for 30 min at room temperature in the dark. Cells were washed and analyzed in a FACScan cytometer using the Cell Quest software.

Immunoblot detection of proteins

Total protein extracts were prepared by lysing cells in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl and 1% NP-

40) for 30 min at 4°C. Proteins were resolved on SDS-PAGE minigels and detected as reported previously.[26] For detection of some nuclear proteins, nuclear extracts were obtained as described previously,[23] and proteins were resolved as above.

Comet assay

DNA damage was quantified using Comet Assay kit (R&D Systems) with some modifications. A total of 1×10^5 cells per milliliter were combined with molten LM agarose at 37°C at a ratio of 1:10 vol/vol and pipetted onto a Comet-Slide. The slides were placed for 10 min in the dark at 4°C before they were immersed in prechilled lysis solution. The slides were then removed from lysis buffer, washed in TBE buffer and transferred to a horizontal electrophoresis chamber. Voltage (1 V/cm) was applied for 20 min. After washing in distilled water, the slides were immersed in 70% ethanol for 5 min and allowed to air dry. Slides were stained with SYBR Green and then analyzed by fluorescence microscopy. Seventy to ninety cells were evaluated in each sample using the Comet Assay Software Project (CASP). DNA damage was quantified by measuring the tail moment (TM) calculated as percentage of DNA in the tail × tail length.

Statistical analysis

The data were analyzed with unpaired Student's *t*-tests (two tailed) by using GraphPad Prism 4 for Windows. Values of $p < 0.05$ were considered significant.

Results

Zebularine and decitabine induce caspase-dependent apoptosis selectively in leukemic T cells

The DNMT inhibitors decitabine and zebularine have been reported to induce apoptosis in different types of cancer cells as a consequence of their demethylating effect.[17,27–30] We analyzed the induction of apoptosis in p53 mutant leukemic T cell lines[31,32] upon treatment for 2 days with decitabine and zebularine, at doses similar to those previously described to induce hypomethylation in leukemia and other tumor cell lines.[28,29,33,34] Jurkat and MOLT-4 cells showed similar sensitivity to both, decitabine and zebularine. Significant apoptosis was observed in both cell lines with all doses used of these compounds, with the exception of 10 μM zebularine (Fig. 1*a*). CEM-6 cells were less sensitive to zebularine than Jurkat and MOLT-4 cells, but concentrations above 10 μM also induced significant apoptosis in this cell line (Fig. 1*a*, right panel). However, CEM-6 cells showed a very low sensitivity to decitabine (Fig. 1*a*, left panel).

Next, we compared the effect of decitabine and zebularine in normal T lymphocytes. Decitabine at all doses used showed a slight and no significant toxicity against both, resting and activated T lymphocytes, whereas zebularine-induced toxicity was only observed at the higher dose of 250 μM (Fig. 1*b*). To further study the effect and the mechanism of action of DNMT inhibitors in leukemia T cell lines, we selected doses of 5 μM decitabine and 50 μM zebularine because of their ability to induce apoptosis in leukemic T cells and their limited toxicity for normal T lymphocytes, especially in the case of zebularine.

Cell cycle analysis performed to determine apoptotic cells in sub-G1 phase revealed that decitabine and zebularine not only differed in their ability to induce apoptosis in different leukemic T cell lines but also in their capacity to affect cell cycle progression. Decitabine induced cell cycle arrest in G2/M phase in Jurkat and CEM-6 cells, but not in MOLT-4 cells, whereas zebularine had no effect on cell cycle distribution (Fig. 1*c*). These observations were confirmed by flow cytometric analysis of the DNA content of viable nonapoptotic cells on a linear scale (Fig. 1*d*). Interestingly, the sensitivity of the different cell lines to both DNMT inhibitors did not correlate with the modulation of cell cycle progression.

Next, we examined the time course of apoptosis induction by decitabine and zebularine in the most sensitive cell lines, Jurkat and MOLT-4 cells. A significant percentage of apoptotic cells was detectable after 30 hr of incubation with either demethylating agent (Figs. 2*a* and 2*b*). In addition, we determined the activation of caspases, as essential components of the apoptotic machinery in mammalian cells. Proteolytic processing of the initiator caspases-8 and -9, as well as cleavage of the effector caspase substrate PARP, was clearly observed as soon as 20 hr after treatment with decitabine and zebularine in both cell lines (Fig. 2*c* and data not shown). Zebularine-induced apoptosis seemed to proceed with faster kinetics than cell death induced by decitabine (Figs. 2*a*–2*c*). To confirm the involvement of caspases in apoptosis triggered by the DNMT inhibitors, we analyzed the effect of the pan-caspase inhibitor Z-VAD-FMK. As shown in Figure 2*d*, apoptosis induced by decitabine and zebularine was completely inhibited by Z-VAD-FMK.

Zebularine and decitabine activate the mitochondrial apoptotic pathway in leukemic T cells

To delineate the mechanism of DNMT inhibitors-induced apoptosis in leukemia T cell lines, we analyzed mitochondrial features of the intrinsic apoptotic pathway. Treatment of Jurkat cells with decitabine and zebularine resulted in loss of $\Delta\psi_m$ and generation of ROS (Fig. 3*a*). Disruption of $\Delta\psi_m$ seemed to precede ROS accumulation as cells in which mitochondria depolarization occurred without ROS production could be detected at 24 hr after treatment. In contrast, all cells producing ROS showed low $\Delta\psi_m$ (Fig. 3*a*). Similar results were found in MOLT-4 cells (data not shown).

The proapoptotic Bcl-2 family members Bax and Bak are essential for the initiation of mitochondrial dysfunction during apoptosis. Because Jurkat cells do not express Bax, we analyzed Bak activation by using an antibody that specifically recognizes its active conformation. As shown in Figure 3*b*, activation of Bak was slightly detected as soon as 20 hr and increased with time of exposure to decitabine and zebularine. In agreement with the kinetics of caspase activation (Fig. 2*c*), time course of Bak activation also suggested that apoptotic

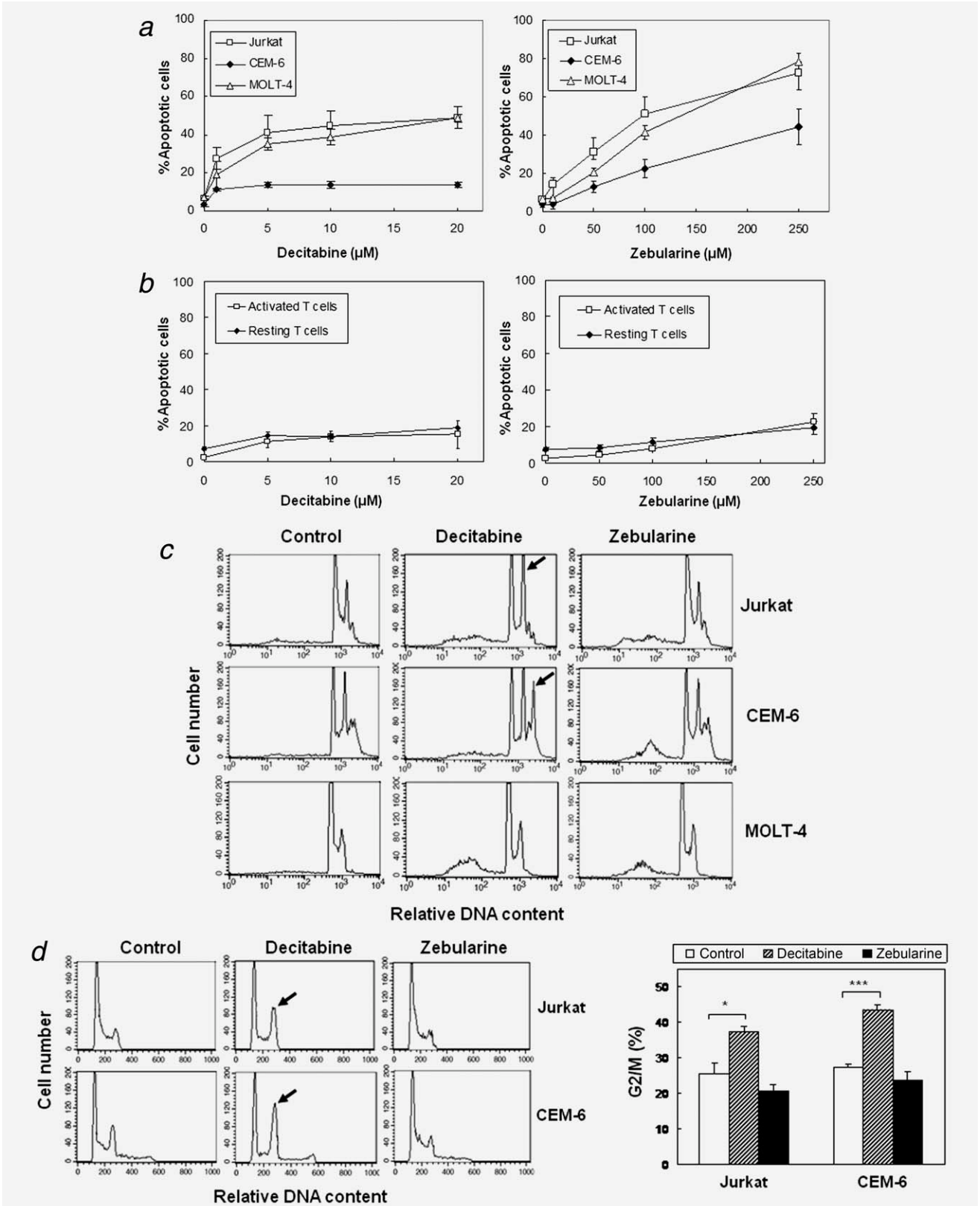


Figure 1. Effects of decitabine and zebularine in apoptosis induction and cell cycle arrest in leukemic T cells. (*a*) Jurkat, CEM-6, MOLT-4, (*b*) resting and activated T cells were treated with different doses of either decitabine (left panels) or zebularine (right panels) for 48 hr. The percentage of sub-G1 (apoptotic) cells was determined by flow cytometry. (*c*) Cell cycle analysis of Jurkat, CEM-6 and MOLT-4 cells, treated without (Control) or with 5 μM decitabine or 50 μM zebularine for 48 hr, was performed by flow cytometry and using logarithmic amplification. (*d*) Cell cycle profile of viable nonapoptotic Jurkat and CEM-6 cells, treated as in *c*, was analyzed by flow cytometry on a linear scale. In the right panel, the percentage of cells in the G2/M phase of the cell cycle was quantified. Error bars show SEM from at least three independent experiments in *a* and *d* and three different donors in *b*. $*p < 0.05$; $**p < 0.01$. In histograms, cells arrested in G2/M are marked by arrows.

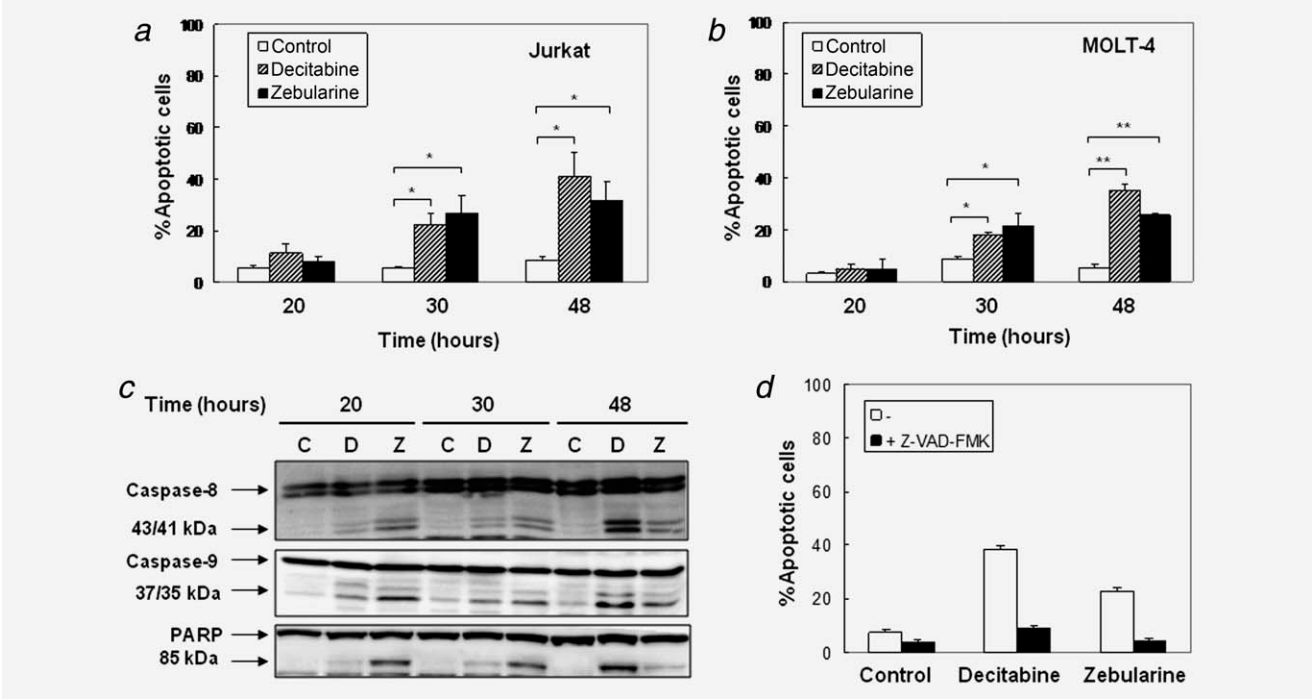


Figure 2. Decitabine and zebularine induce caspase-dependent apoptosis in leukemic T cells. (*a*) Jurkat and (*b*) MOLT-4 cells were treated without (control) or with 5 μM decitabine or 50 μM zebularine for 20, 30 and 48 hr, and the percentage of sub-G1 cells was determined by flow cytometry. (*c*) Activation of caspases-8 and -9 and PARP degradation were determined by Western blot in Jurkat cells treated as in *a* (C: control; D: decitabine; Z: zebularine). (*d*) Jurkat cells were preincubated for 1 hr in the absence or in the presence of the caspase inhibitor Z-VAD-FMK (20 μM) and then treated for 48 hr with 5 μM decitabine or 50 μM zebularine. Sub-G1 cells were analyzed by flow cytometry. Error bars show SEM from three independent experiments. *$p < 0.05$; **$p < 0.01$.

signals are more rapidly triggered in response to zebularine (Fig. 3*b*).

The involvement of mitochondria in apoptosis induced by DNMT inhibitors was further supported by examining the effect of decitabine and zebularine in Jurkat cells overexpressing Bcl-2 or Bcl-x_L. We observed that overexpression of these antiapoptotic proteins completely prevented the appearance of sub-G1 apoptotic cells and the activation of caspases in response to treatment with DNMT inhibitors when compared to mock-transfected Jurkat cells (Figs. 3*c* and 3*d*). The existence of different Jurkat subclones that derive from different sources may explain the lower sensitivity to decitabine of mock-transfected Jurkat cells in comparison with the Jurkat cells used above (Figs. 1*a* and 3*c*). We also demonstrated that decitabine and zebularine did not induce loss of $\Delta\psi_m$ and ROS generation in Bcl-2- and Bcl-x_L-overexpressing Jurkat cells (Fig. 3*e*).

ROS production is involved in zebularine- and decitabine-induced apoptosis in leukemic T cells

To establish whether ROS production could play a role in the induction of cell death by DNA demethylating agents, the effect of the cell-permeable superoxide dismutase (SOD) mimetic manganese-porphyrin, Mn(III)TMPyP, was analyzed. As shown in Figure 4*a*, apoptosis induced by decitabine and zebularine was almost completely blocked in cells pretreated with the SOD mimetic, suggesting that this mode of cell death depends on the production of ROS. We confirmed that the Mn-porphyrin inhibited ROS generation in response to treatment with both demethylating agents (Fig. 4*b*). Interestingly, loss of $\Delta\psi_m$ induced by DNTM inhibitors was not prevented by the SOD mimetic, thus confirming our hypothesis that this mitochondrial event preceded the accumulation of ROS.

Caspase-9 is essential for zebularine- and decitabine-induced apoptosis in Jurkat cells

Initiator caspase-9 is a critical component of the mitochondrial pathway of apoptosis. To ascertain the requirement of caspase-9 in the induction of apoptosis by DNMT inhibitors, we studied the response of a caspase-9-deficient Jurkat clone to these agents. Neither decitabine nor zebularine was able to induce apoptosis in caspase-9-deficient Jurkat cells (Jurkat w/o C9, Fig. 5*a*). As a control, induction of apoptosis was determined in deficient cells stably transfected with a caspase-9 expression vector (Jurkat C9, Fig. 5*a*). In addition, we analyzed the mitochondrial events characterized above. Decitabine and zebularine induced a weak activation of Bak in caspase-9-deficient cells when compared to reconstituted cells (Fig. 5*b*). However, both demethylating agents failed to

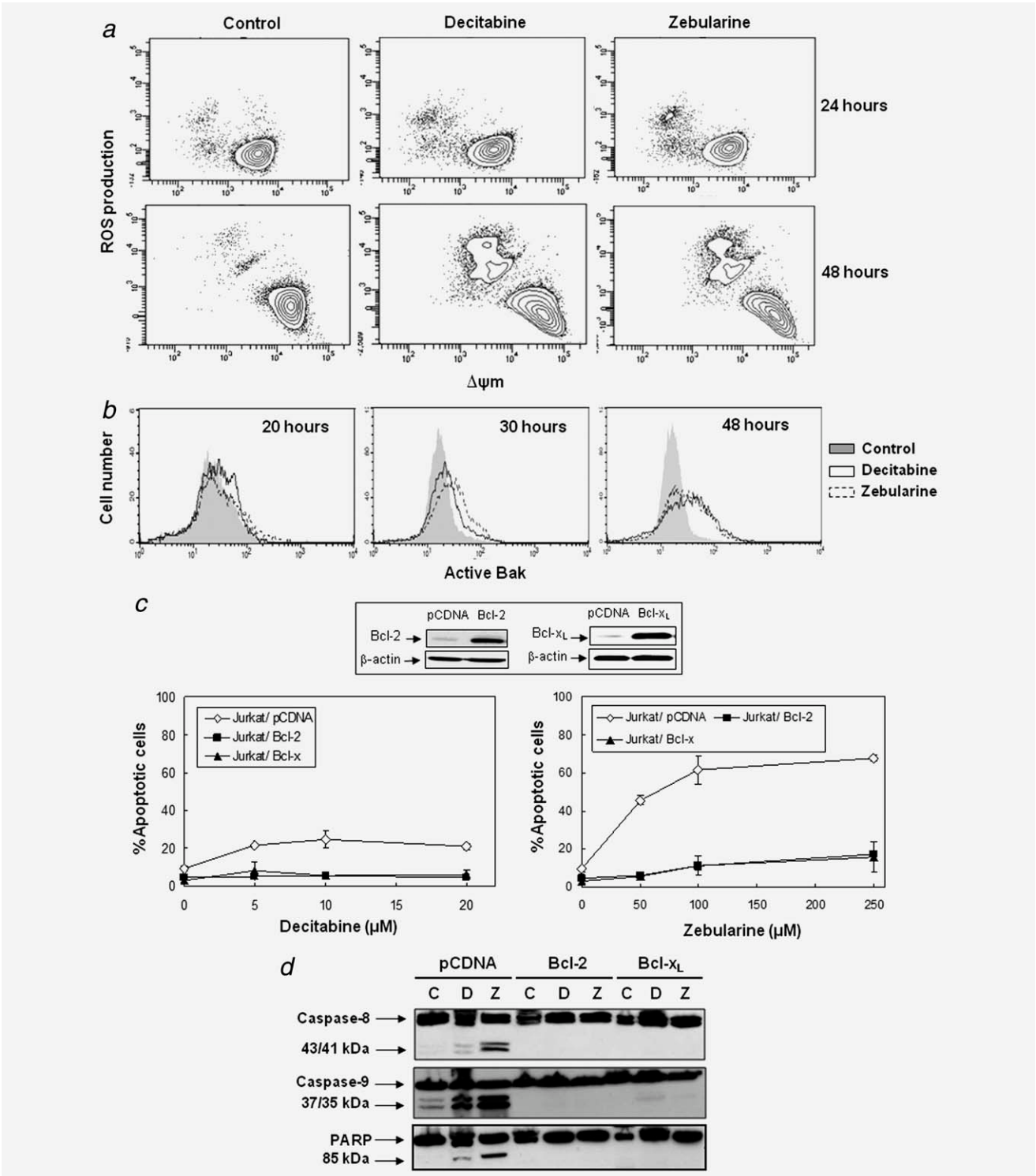


Figure 3. Induction of mitochondrial apoptotic events in leukemic T cells by decitabine and zebularine. (*a*) Jurkat cells were treated without (control) or with 5 μM decitabine or 50 μM zebularine for 24 and 48 hr. Mitochondrial membrane potential ($\Delta\psi_m$) and ROS production were determined by flow cytometry using $DiOC_6(3)$ and dihydroethidium, respectively. (*b*) Bak activation was analyzed by flow cytometry in Jurkat cells treated for the indicated times as in *a*. (*c*) Mock-transfected (Jurkat/pCDNA), Bcl-2-overexpressing (Jurkat/Bcl-2) and Bcl-x_L-overexpressing (Jukat/Bcl-x_L) Jurkat cells were treated with the indicated doses of either decitabine (left panel) or zebularine (right panel) for 48 hr. Sub-G1 cells were determined by flow cytometry. Inset shows levels of Bcl-2, Bcl-x_L and β-actin in mock-transfected, Bcl-2- and Bcl-x_L-overexpressing Jurkat cells, as determined by Western blot. (*d*) Mock-transfected, Bcl-2- and Bcl-x_L-overexpressing Jurkat cells were incubated with 5 μM decitabine and 50 μM zebularine for 48 hr. Caspases-8 and -9 activation and PARP degradation were analyzed by Western blot (C: control; D: decitabine; Z: zebularine). (*e*) Mitochondrial membrane potential and ROS production were determined by flow cytometry in mock-transfected, Bcl-2- and Bcl-x_L-overexpressing Jurkat cells treated as in *d*. Error bars in *c* represent SEM from three independent experiments.

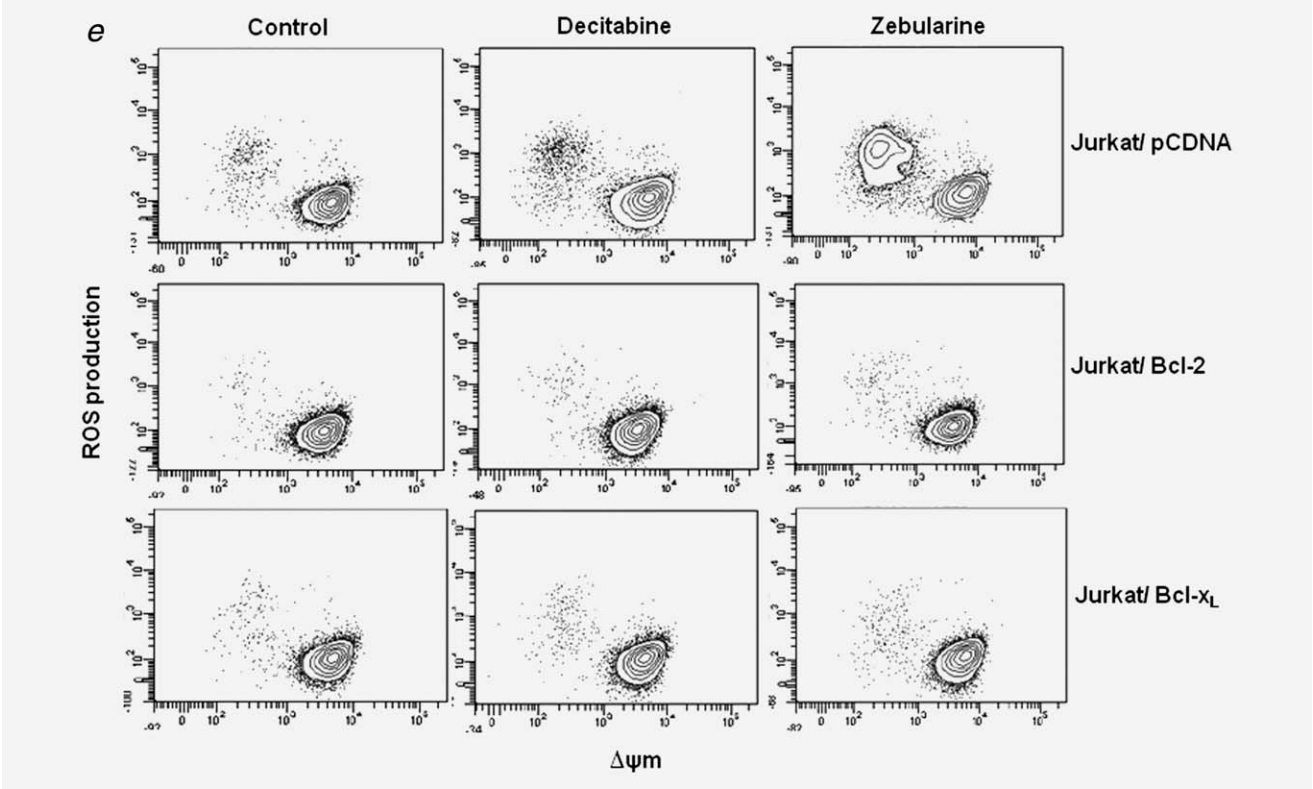


Figure 3. (Continued)

induce disruption of $\Delta\psi_m$ and ROS accumulation in caspase-9-lacking Jurkat cells (Fig. 5*c*). These results imply that caspase-9 is necessary to fully promote the mitochondrial events that lead to apoptotic cell death.

Zebularine and decitabine induce DNA damage in leukemic T cells

Because induction of DNA strand breaks by nucleoside DNMT inhibitors has been implicated in their cytotoxic effects in other tumor models,[15] we assessed, by comet assay, the ability of decitabine and zebularine to induce DNA damage in leukemic T cells. Following gel electrophoresis, comet tails indicative of DNA damage were detected in Jurkat cells treated with decitabine and zebularine (Fig. 6*a*, upper panel). Calculation of the TM in time-course experiments indicated that cells with DNA strand breaks began to accumulate between 20 and 30 hr after addition of DNMT inhibitors (Fig. 6*a*, lower left panel). A similar experiment performed in MOLT-4 cells showed that DNA breaks were evident at 20 hr after treatment, although the level of damage accumulated was lower than that in Jurkat cells at longer times (Fig. 6*a*, lower right panel). Interestingly, the amount of DNA damage was higher in response to zebularine in all cases. We also analyzed phosphorylation of the histone H2AX (γ-H2AX), as an early marker of DNA damage. Incubation with either decitabine or zebularine resulted in a time-dependent increase in γ-H2AX levels, already evident at 20 hr (Fig. 6*b*). Moreover, we determined the activation of two proteins involved in DNA damage response, *i.e.*, the ATM and ATR downstream substrate Chk1 and the DNA damage sensor Nbs1.[35] The active, phosphorylated forms of Chk1 and Nbs1 were clearly detected at all time points analyzed in response to decitabine, whereas they were more weakly induced by zebularine (Fig. 6*b*). Similar results were found in both, Jurkat and MOLT-4 cells. On the other hand, when we analyzed DNMT1 depletion, indicative of the hypomethylating effect of those inhibitors, we observed that it was apparent after more prolonged incubation (around 30 hr) and almost complete at 48 hr (Fig. 6*b*). The expression of DNMT1 protein has been reported to be cell replication dependent,[33] which may explain the observed differences in basal levels of enzyme at different time points in Jurkat cells. Taken together, these results suggest that induction of DNA damage by decitabine and zebularine might play a major role in their cytotoxic activity against leukemic T cells.

Finally, we analyzed the induction of DNA strand break and the activation of DNA damage response in the less sensitive cell line CEM-6. Comet assay showed that decitabine and zebularine induced a similar accumulation of cells with DNA strand breaks that was already evident at 20 hr after treatment (Fig. 6*c*, left panel). In addition, both DNTM inhibitors induced phosphorylation of H2AX, Chk1 and Nbs1 at all times analyzed, being more intensive in response to decitabine at the longer time points (Fig. 6*c*, right panel).

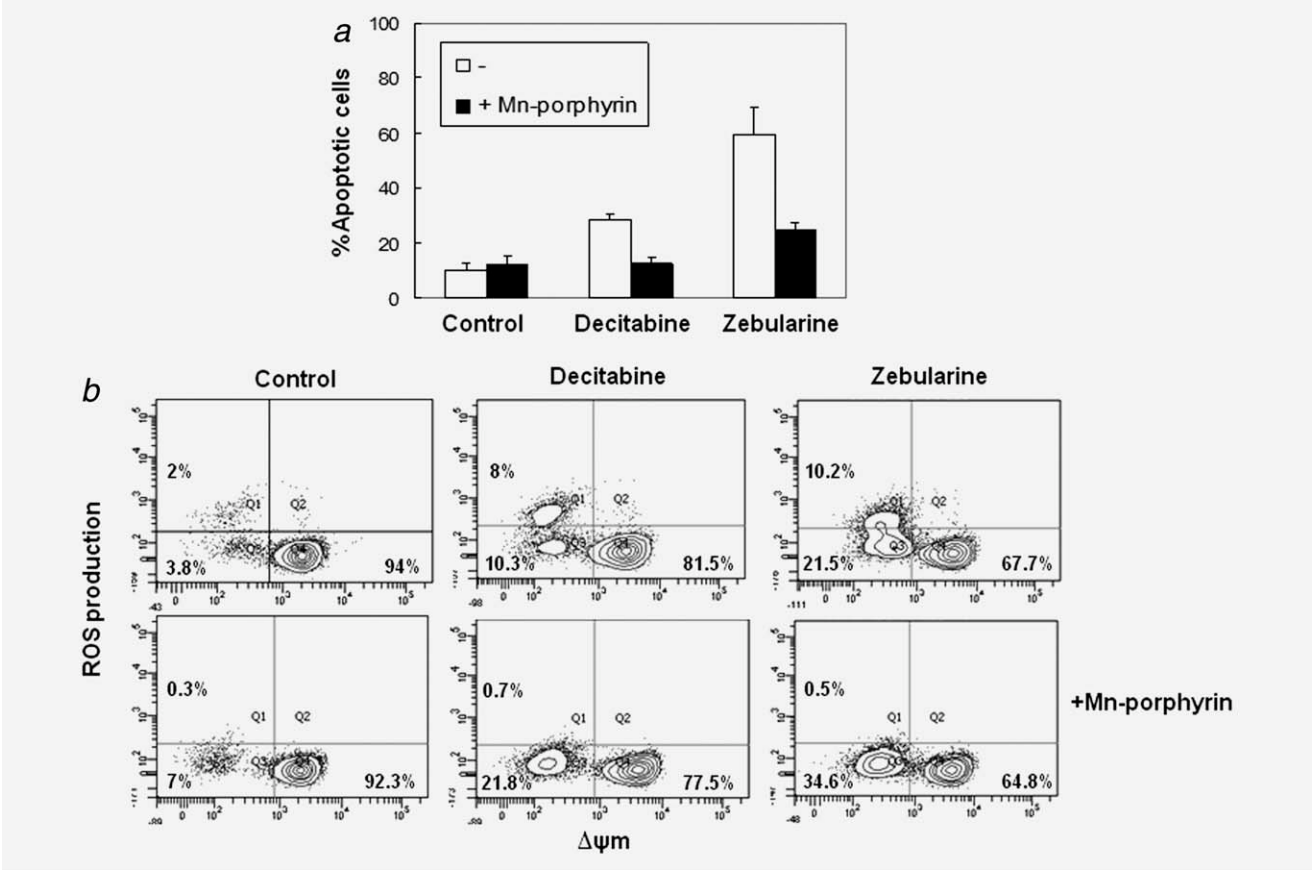


Figure 4. ROS scavenging prevents decitabine- and zebularine-induced apoptosis in leukemic T cells. Jurkat cells were preincubated for 24 hr in the absence or in the presence of 250 μM manganese porphyrin Mn(III)TMPyP and then treated without (control) or with 5 μM decitabine or 50 μM zebularine for 48 hr. (*a*) Hypodiploid apoptotic cells, (*b*) mitochondrial membrane potential and ROS production were determined by flow cytometry. Error bars in *a* show SEM from three independent experiments.

Discussion

DNMT inhibiting nucleoside analogs have been recently proposed as efficient drugs for the treatment of hematologic malignancies. The promising activity of decitabine has been proven in multiple clinical trials in MDS, chronic and acute myeloid leukemias.[6,36] In addition, demethylating effects and induction of apoptosis in response to treatment with zebularine have been demonstrated in MLL-rearranged infant acute lymphoblastic leukemia and in acute myeloblastic leukemia.[28,29] However, the mechanism of antitumor action of these drugs is incompletely understood. Their ability to reactivate the expression of tumor suppressor genes and to induce apoptosis seems to depend not only on their demethylating activity but also on their DNA-damaging effect. It also remains unclear which apoptotic pathway is activated by nucleoside DNMT inhibitors. For our study, we set out to examine the ability of decitabine and zebularine to selectively induce apoptosis in leukemic T cells and the mechanism of apoptosis induction by these demethylating agents.

Recent studies have indicated a resistance of peripheral blood mononuclear cells to cell death induced by decitabine.[17,37] However, to our knowledge, our results are the first to show the high resistance of not only resting but also activated and hence proliferating T cells to decitabine- and zebularine-induced apoptosis. We demonstrate here that both decitabine and zebularine, at doses that show no significant toxicity for normal T lymphocytes, induce caspase-dependent mitochondria-mediated apoptosis in leukemic T cell lines. These are interesting results as there are no previous reports describing the signaling pathway of zebularine-induced apoptosis. Moreover, although decitabine has been more extensively studied, controversial results exist about the role of caspases in the induction of apoptosis by this DNMT inhibitor. Although several authors have shown the activation of effector caspases, *i.e.*, caspase-3, in response to decitabine, the activation of initiator caspases and the overall requirement of caspase activation for the induction of apoptosis is unclear.[17,30,38,39] Our data clearly show that decitabine and zebularine induce the activation of caspase-9 and effector caspases, as demonstrated by the cleavage of their substrate PARP. In addition, apoptosis was completely inhibited by the pan-caspase inhibitor Z-VAD-FMK as well as by the

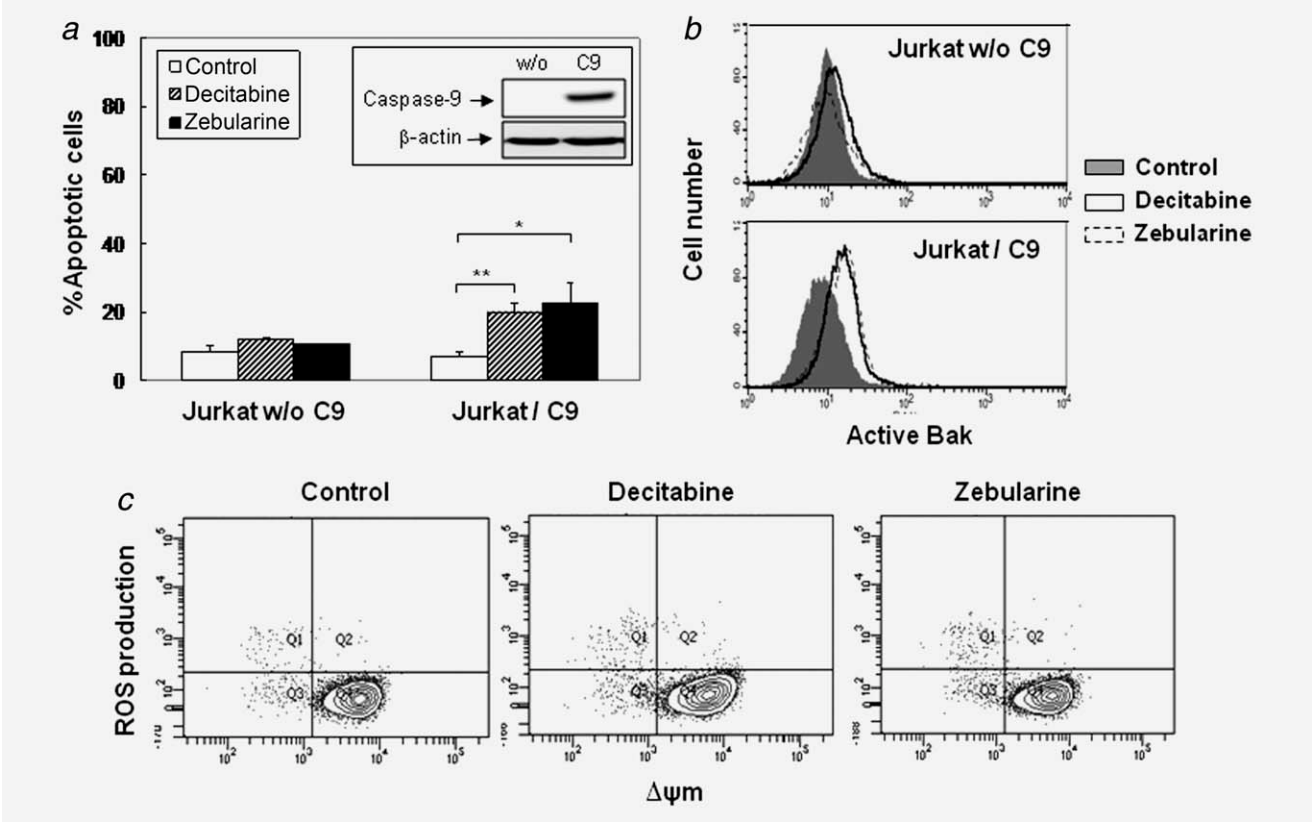


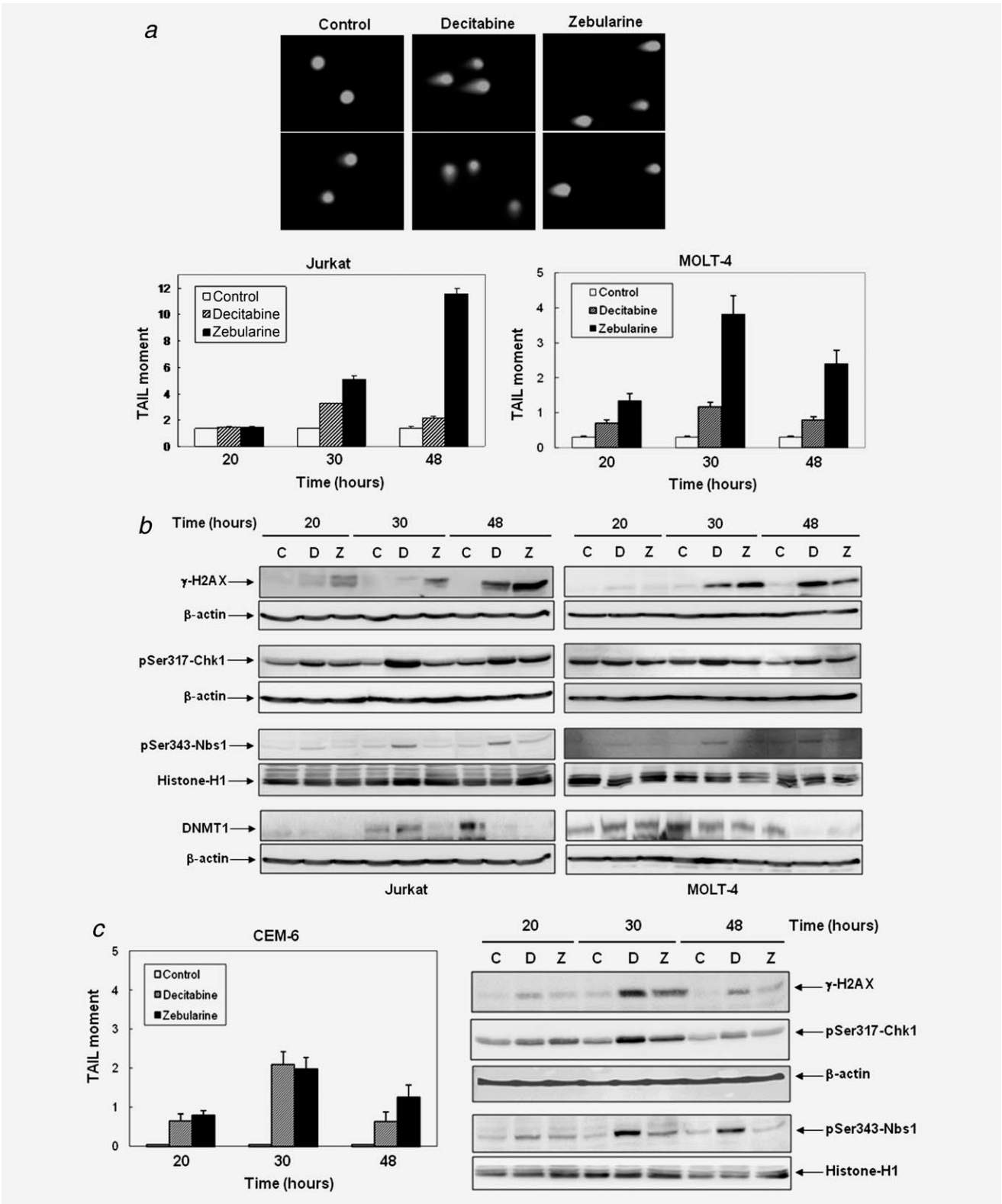
Figure 5. Caspase-9-deficient Jurkat cells are resistant to the induction of apoptosis by decitabine and zebularine. (*a*) Sub-G1 apoptotic cells were determined by flow cytometry in caspase-9-deficient (Jurkat w/o C9) and caspase-9-reconstituted (Jurkat/C9) Jurkat cells after incubation without (control) or with 5 μM decitabine or 50 μM zebularine for 48 hr. Error bars show SEM from three independent experiments. $*p < 0.05$; $**p < 0.01$. The inset figure shows the levels of caspase-9 and β-actin in caspase-9-lacking and -reconstituted cells as determined by Western blot. (*b*) Caspase-9-deficient and caspase-9-reconstituted Jurkat cells were treated as in *a*, and Bak activation was analyzed by flow cytometry. (*c*) Mitochondrial membrane potential and ROS production were determined by flow cytometry in caspase-9-deficient Jurkat cells treated as in *a*.

deficiency of caspase-9, further demonstrating that caspase activation is necessary for cell death induction by these demethylating agents.

Regarding the involvement of mitochondria, some authors have shown that treatment with decitabine results in dissipation of transmembrane potential.[17,30] Similarly, we have observed the loss of $\Delta\psi_m$ in response to decitabine and also to zebularine. Moreover, we have shown that both DNMT inhibitors induce Bak activation and ROS production but do not trigger apoptosis in Jurkat cells overexpressing either Bcl-2 or Bcl-x_L. These results, together with the observed resistance of caspase-9-deficient Jurkat cells, confirm the crucial role of mitochondria in apoptosis induced by decitabine and zebularine. Previous studies with caspase-9-lacking cells have demonstrated that loss of $\Delta\psi_m$ in response to genotoxic stress depends on caspase-9 activation.[24] In accordance with these results, we demonstrate that in the absence of caspase-9 there are no mitochondrial apoptotic events, with the exception of a weak activation of Bak. Our findings support a model in which the initial mitochondrial outer membrane permeabilization, mediated by proapoptotic Bcl-2 proteins such as Bak, allows the release of intermembrane space proteins and the subsequent activation of caspase-9 and effector caspases. Subsequently, downstream caspases elicit feedback amplification on the permeabilized mitochondria to promote the disruption of mitochondrial function.[24,40,41] We have further shown that production of ROS follows the loss of $\Delta\psi_m$ in leukemic T cells upon treatment with decitabine and zebularine. In spite of the fact that ROS generation is a secondary mitochondrial event in this model, apoptosis was prevented by ROS scavenging with a SOD mimetic, suggesting that caspase-dependent ROS production plays a crucial role in the dismantling of the cell.

The above data lead us to state that the mechanism of cell death induces by nucleoside DNMT inhibitors is comparable to that induce by genotoxic stress. In fact, upon exposure to decitabine and zebularine, DNA damage was observed in leukemic T cell lines, as determined by comet assay,

accumulation of γ-H2AX and activation of proteins of the DNA damage response pathway, such as Nbs1 and Chk1.[35] Kinetics of caspase activation and disruption of mitochondria correlated with that of H2AX phosphorylation and activation of DNA repair proteins and preceded depletion of DNMT1. Consistent with our results, several reports have suggested



that molecular mechanisms other than reexpression of methylated genes contribute to the antitumor activity of decitabine, one such mechanism being the induction of DNA damage.[16,42–44] Interestingly, these authors indicate that the effects of decitabine on cellular proliferation and viability are mediated by the upregulation of p21 in a p53-dependent manner.[16,43,44] However, leukemic T cell lines used in our study do not have functional p53, and we did not observe changes in the levels of p21 in response to DNMT inhibitors (data not shown). A similar finding was described by Nieto *et al.* who demonstrated that primary and transformed mouse embryonic fibroblasts deficient for p53 are hypersensitive to decitabine.[45] It is possible that the role of p53 depends on the cell type and the presence of other molecules that may substitute for p53 function, such as p73.[17] On the other hand, p53 mutant leukemic T cells, *i.e.*, Jurkat cells, have been previously reported to be sensitive to genotoxic drugs, suggesting that, at least in this cell model, DNTM inhibitors might induce apoptosis by a p53-independent mechanism similar to that of genotoxic stress.[24,40]

Concerning zebularine, most studies have focused on its activity as a DNMT inhibitor, but few data exist about its ability to induce apoptosis, cell growth arrest and DNA damage.[15,28,34] Here, we have demonstrated that zebularine is able to induce apoptosis in leukemia T cell lines in a similar way to decitabine. However, we have to underline that there must be some differences in the mechanism of action between zebularine and decitabine, even though both drugs finally trigger the same apoptotic signaling pathway. For instance, zebularine did not induce cell cycle arrest in leukemic T cells, in contrast to decitabine which arrests Jurkat and CEM-6, but not MOLT-4 cells, in G2/M. The ability of decitabine to induce cell cycle arrest has been associated with activation of the p53 pathway,[15,16] but this cannot explain the arrest in our p53 mutant cell lines. However, it may be associated with activation of Chk1, a central regulator of the S and G2 checkpoint response. Chk1 induces the inhibition of cyclin-dependent kinases (Cdk) leading to cell cycle arrest.[35] We have shown that activation (phosphorylation) of Chk1 is more significant in response to decitabine in Jurkat and CEM-6 cells, which could explain the arrest in G2/M phase by this inhibitor. One can speculate that the lower activation of Chk1 by decitabine in MOLT-4 cells should be insufficient to halt cell cycle. Another important difference between decitabine and zebularine lies in their ability to induce apoptosis in CEM-6 cells. This could also be related with G2/M arrest as it increases the time available for DNA repair, thus favoring cell survival. In contrast, unrepaired DNA strand breaks can easily lead to cell death. Interestingly, it has been reported that zebularine enhances radiosensitivity by inhibition of DNA repair, although the mechanism is not known.[46] The weak activation of DNA repair proteins (Nbs1 and Chk1) that we have observed in response to zebularine would be in agreement with this report. Clearly, additional studies are required to further understand the differential effects of decitabine and zebularine on DNA damage response as well as the differences in susceptibility among cell lines to both demethylating agents.

Comparative studies have revealed that zebularine is much less effective than decitabine as a hypomethylating agent and have shown different effects of both agents on gene transcription.[47,48] In agreement with our suggestion, the authors conclude that the mechanism of action of decitabine and zebularine is not identical, and their effects may at least in part be independent of DNA demethylation.[47] Interestingly, zebularine is also a potent inhibitor of cytidine deaminase, and, because of this property, it has been suggested to improve the antitumor activity of decitabine when both drugs are coadministered,[49,50] although we did not observe any synergistic effect on leukemic T cell lines after a combined treatment with decitabine and zebularine (data not shown). On the other hand, zebularine is highly stable in aqueous solution, and, even more importantly, it exerts a lower toxicity than decitabine.[7,8] It was demonstrated that zebularine is relatively selective for cancer cells that incorporate this drug more efficiently into DNA when compared to normal cells.[9] Incorporation of zebularine into DNA requires its phosphorylation by the uridine-cytidine kinase (UCK).[50] There are two human UCKs, UCK1 and UCK2, but UCK activity closely correlates with levels of UCK2.[51] The fact that UCK2 is mainly expressed in tumor cells but not in normal tissues[51] may explain the more efficient incorporation of zebularine to cancer cells than to normal cells.

In preliminary studies with two samples of primary T-cell lymphoblastic leukemia, we observed that zebularine induced a higher level of apoptosis than decitabine (data not shown).

Figure 6. Treatment of leukemic T cells with decitabine and zebularine results in DNA damage. (*a*) DNA strand breaks were analyzed by comet assay in Jurkat and MOLT-4 cells treated without (control) or with 5 μM decitabine or 50 μM zebularine either for 48 hr (upper panel) or for the indicated times (lower panels). Images of Jurkat cells with comet tails are shown on the upper panel. The comet tail moments were scored from at least 70 cells per sample (mean ± SEM, lower panels). (*b*) Expression of γ-H2AX, phospho-Chk1 (Ser317), phospho-Nbs1 (Ser343) and DNMT1 was analyzed by Western blot in Jurkat and MOLT-4 cells in response to treatment without (C) or with 5 μM decitabine (D) or 50 μM zebularine (Z) for the indicated times. β-Actin and histone-H1 were used as loading controls for total and nuclear extracts, respectively. (*c*) CEM cells were treated without (C) or with 5 μM decitabine (D) or 50 μM zebularine (Z) for the indicated times. DNA strand breaks were analyzed by comet assay, and tail moments were scored from at least 70 cells per sample (mean ± SEM, left panel). Expression of γ-H2AX, phospho-Chk1 (Ser317) and phospho-Nbs1 (Ser343) was analyzed by Western blot, and loading controls were used as in *b* (right panel). Data shown are representative of two independent experiments.

Overall, these data, together with the fast kinetics of apoptosis induction and the sensitivity of leukemic T cell lines, suggest that zebularine could be the one of choice for the treatment of T-cell leukemias.

Acknowledgements

The authors thank Dr. F. Javier Oliver for invaluable advice and generous donation of reagents. This work was supported by the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo (C.R.-R.).

References

1. Esteller M. Aberrant DNA methylation as a cancer-inducing mechanism. *Annu Rev Pharmacol Toxicol* 2005;45:629–56.
2. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349: 2042–54.
3. Baylin SB. DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol* 2005;2 (Suppl 1):S4–S11.
4. Christman JK. 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 2002;21:5483–95.
5. Stresemann C, Lyko F. Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer* 2008;123:8–13.
6. Kuendgen A, Lubbert M. Current status of epigenetic treatment in myelodysplastic syndromes. *Ann Hematol* 2008; 87:601–11.
7. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, Jones PA, Selker EU. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J Natl Cancer Inst* 2003;95: 399–409.
8. van Bemmel DM, Brank AS, Eritja R, Marquez VE, Christman JK. DNA (Cytosine-C5) methyltransferase inhibition by oligodeoxyribonucleotides containing 2-(1H)-pyrimidinone (zebularine aglycon) at the enzymatic target site. *Biochem Pharmacol* 2009;78:633–41.
9. Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, Marquez VE, Greer S, Orntoft TF, Thykjaer T, Jones PA. Preferential response of cancer cells to zebularine. *Cancer Cell* 2004;6:151–8.
10. Boultwood J, Wainscoat JS. Gene silencing by DNA methylation in haematological malignancies. *Br J Haematol* 2007;138: 3–11.
11. Fu WN, Bertoni F, Kelsey SM, McElwaine SM, Cotter FE, Newland AC, Jia L. Role of DNA methylation in the suppression of Apaf-1 protein in human leukaemia. *Oncogene* 2003;22:451–5.
12. Fulda S, Kufer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B, Debatin KM. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene* 2001;20: 5865–77.
13. Sigalotti L, Fratta E, Coral S, Cortini E, Covre A, Nicolay HJM, Anzalone L, Pezzani L, Di Giacomo AM, Fonsatti E, Colizzi F, Altomonte M, et al. Epigenetic drugs as pleiotropic agents in cancer treatment: biomolecular aspects and clinical applications. *J Cell Physiol* 2007; 212:330–44.
14. Kiziltepe T, Hideshima T, Catley L, Raje N, Yasui H, Shiraishi N, Okawa Y, Ikeda H, Vallet S, Pozzi S, Ishitsuka K, Ocio EM, et al. 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. *Mol Cancer Ther* 2007;6:1718–27.
15. Palii SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD. DNA methylation inhibitor 5-aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. *Mol Cell Biol* 2008;28:752–71.
16. Jiemjit A, Fandy TE, Carraway H, Bailey KA, Baylin S, Herman JG, Gore SD. p21(WAF1/CIP1) induction by 5-azacytosine nucleosides requires DNA damage. *Oncogene* 2008;27:3615–23.
17. Schmelz K, Wagner M, Dorken B, Tamm I. 5-Aza-2′-deoxycytidine induces p21WAF expression by demethylation of p73 leading to p53-independent apoptosis in myeloid leukemia. *Int J Cancer* 2005; 114:683–95.
18. Tamm I, Wagner M, Schmelz K. Decitabine activates specific caspases downstream of p73 in myeloid leukemia. *Ann Hematol* 2005;84 (Suppl 1):47–53.
19. Kuang SQ, Tong WG, Yang H, Lin W, Lee MK, Fang ZH, Wei Y, Jelinek J, Issa JP, Garcia-Manero G. Genome-wide identification of aberrantly methylated promoter associated CpG islands in acute lymphocytic leukemia. *Leukemia* 2008;22: 1529–38.
20. Taylor KH, Pena-Hernandez KE, Davis JW, Arthur GL, Duff DJ, Shi H, Rahmatpanah FB, Sjahputera O, Caldwell CW. Large-scale CpG methylation analysis identifies novel candidate genes and reveals methylation hotspots in acute lymphoblastic leukemia. *Cancer Res* 2007; 67:2617–25.
21. Yasunaga J, Taniguchi Y, Nosaka K, Yoshida M, Satou Y, Sakai T, Mitsuya H, Matsuoka M. Identification of aberrantly methylated genes in association with adult T-cell leukemia. *Cancer Res* 2004;64: 6002–9.
22. Milani L, Lundmark A, Kiialainen A, Nordlund J, Flaegstad T, Forestier E, Heyman M, Jonmundsson G, Kanerva J, Schmiegelow K, Soderhall S, Gustafsson MG, et al. DNA methylation for subtype classification and prediction of treatment outcome in patients with childhood acute lymphoblastic leukemia. *Blood* 2010;115: 1214–25.
23. Morales JC, Ruiz-Magaña MJ, Ruiz-Ruiz C. Regulation of the resistance to TRAIL-induced apoptosis in human primary T lymphocytes: role of NF-kappaB inhibition. *Mol Immunol* 2007;44:2587–97.
24. Samraj AK, Sohn D, Schulze-Osthoff K, Schmitz I. Loss of caspase-9 reveals its essential role for caspase-2 activation and mitochondrial membrane depolarization. *Mol Biol Cell* 2007;18:84–93.
25. Gong J, Traganos F, Darzynkiewicz Z. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal Biochem* 1994; 218:314–19.
26. Ruiz-Ruiz MC, Lopez-Rivas A. p53-mediated up-regulation of CD95 is not involved in genotoxic drug-induced apoptosis of human breast tumor cells. *Cell Death Differ* 1999;6:271–80.
27. Kubo T, Yamamoto J, Shikauchi Y, Niwa Y, Matsubara K, Yoshikawa H. Apoptotic speck protein-like, a highly homologous protein to apoptotic speck protein in the pyrin domain, is silenced by DNA methylation and induces apoptosis in human hepatocellular carcinoma. *Cancer Res* 2004;64:5172–7.
28. Scott SA, Lakshimikuttysamma A, Sheridan DP, Sanche SE, Geyer CR, DeCoteau JF. Zebularine inhibits human acute myeloid leukemia cell growth in vitro in association with p15INK4B demethylation and reexpression. *Exp Hematol* 2007; 35:263–73.
29. Stumpel DJ, Schneider P, van Roon EH, Boer JM, de Lorenzo P, Valsecchi MG, de Menezes RX, Pieters R, Stam RW. Specific promoter methylation identifies different subgroups of MLL-rearranged infant acute lymphoblastic leukemia, influences clinical outcome, and provides therapeutic options. *Blood* 2009;114:5490–8.

30. Khan R, Schmidt-Mende J, Karimi M, Gogvadze V, Hassan M, Ekstrom TJ, Zhivotovsky B, Hellstrom-Lindberg E. Hypomethylation and apoptosis in 5-azacytidine-treated myeloid cells. *Exp Hematol* 2008;36:149–57.
31. Cheng J, Haas M. Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. *Mol Cell Biol* 1990; 10:5502–9.
32. Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP. p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990;87:7555–9.
33. Qin T, Jelinek J, Si J, Shu J, Issa JP. Mechanisms of resistance to 5-aza-2′-deoxycytidine in human cancer cell lines. *Blood* 2009;113:659–67.
34. Billam M, Sobolewski MD, Davidson NE. Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. *Breast Cancer Res Treat* 2010; 120:581–92.
35. Al-Ejeh F, Kumar R, Wiegmans A, Lakhani SR, Brown MP, Khanna KK. Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes. *Oncogene* 2010; 29:6085–9.
36. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H. Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer* 2008;112:2341–51.
37. Yan P, Qu Z, Ishikawa C, Mori N, Xiao G. Human T-cell leukemia virus type I-mediated repression of PDZ-LIM domain-containing protein 2 involves DNA methylation but independent of the viral oncoprotein tax. *Neoplasia* 2009;11: 1036–41.
38. Deng T, Zhang Y. Possible involvement of activation of P53/P21 and demethylation of RUNX 3 in the cytotoxicity against Lovo cells induced by 5-Aza-2′-deoxycytidine. *Life Sci* 2009;84:311–20.
39. Hoglund A, Nilsson LM, Forshell LP, Maclean KH, Nilsson JA. Myc sensitizes p53-deficient cancer cells to the DNA-damaging effects of the DNA methyltransferase inhibitor decitabine. *Blood* 2009;113:4281–8.
40. Franklin EE, Robertson JD. Requirement of Apaf-1 for mitochondrial events and the cleavage or activation of all procaspases during genotoxic stress-induced apoptosis. *Biochem J* 2007;405: 115–22.
41. Ricci JE, Munoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N, Scheffler IE, Ellisman MH, Green DR. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* 2004;117: 773–86.
42. Schmelz K, Sattler N, Wagner M, Lubbert M, Dorken B, Tamm I. Induction of gene expression by 5-Aza-2′-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. *Leukemia* 2005;19:103–11.
43. Zhu WG, Hileman T, Ke Y, Wang P, Lu S, Duan W, Dai Z, Tong T, Villalona-Calero MA, Plass C, Otterson GA. 5-aza-2′-deoxycytidine activates the p53/p21Waf1/Cip1 pathway to inhibit cell proliferation. *J Biol Chem* 2004;279: 15161–6.
44. Pulukuri SM, Rao JS. Activation of p53/p21Waf1/Cip1 pathway by 5-aza-2′-deoxycytidine inhibits cell proliferation, induces pro-apoptotic genes and mitogen-activated protein kinases in human prostate cancer cells. *Int J Oncol* 2005;26: 863–71.
45. Nieto M, Samper E, Fraga MF, Gonzalez de Buitrago G, Esteller M, Serrano M. The absence of p53 is critical for the induction of apoptosis by 5-aza-2′-deoxycytidine. *Oncogene* 2004;23:735–43.
46. Dote H, Cerna D, Burgan WE, Carter DJ, Cerra MA, Hollingshead MG, Camphausen K, Tofilon PJ. Enhancement of in vitro and in vivo tumor cell radiosensitivity by the DNA methylation inhibitor zebularine. *Clin Cancer Res* 2005;11:4571–9.
47. Flotho C, Claus R, Batz C, Schneider M, Sandrock I, Ihde S, Plass C, Niemeyer CM, Lubbert M. The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells. *Leukemia* 2009;23: 1019–28.
48. Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res* 2006; 66:2794–800.
49. Lemaire M, Momparler LF, Raynal NJ, Bernstein ML, Momparler RL. Inhibition of cytidine deaminase by zebularine enhances the antineoplastic action of 5-aza-2′-deoxycytidine. *Cancer Chemother Pharmacol* 2009;63:411–16.
50. Marquez VE, Kelley JA, Agbaria R, Ben-Kasus T, Cheng JC, Yoo CB, Jones PA. Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. *Ann N Y Acad Sci* 2005; 1058:246–54.
51. Shimamoto Y, Koizumi K, Okabe H, Kazuno H, Murakami Y, Nakagawa F, Matsuda A, Sasaki T, Fukushima M. Sensitivity of human cancer cells to the new anticancer ribo-nucleoside TAS-106 is correlated with expression of uridine-cytidine kinase 2. *Jpn J Cancer Res* 2002;93:825–33.