Cladribine induces apoptosis in human leukaemia cells by caspase-dependent and -independent pathways acting on mitochondria

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We have studied the role of caspases and mitochondria in apoptosis induced by 2-chloro-2′-deoxyadenosine (cladribine) in several human leukaemic cell lines. Cladribine treatment induced mitochondrial transmembrane potential ($\Delta\Psi_m$) loss, phosphatidylserine exposure, caspase activation and development of typical apoptotic morphology in JM1 (pre-B), Jurkat (T) and U937 (promonocytic) cells. Western-blot analysis of cell extracts revealed the activation of at least caspases 3, 6, 8 and 9. Co-treatment with Z-VAD-fmk (benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone), a general caspase inhibitor, significantly prevented cladribine-induced death in JM1 and Jurkat cells for the first $\approx$ 40 h, but not for longer times. Z-VAD-fmk also partly prevented some morphological and biochemical features of apoptosis in U937 cells, but not cell death. Co-incubation with selective caspase inhibitors Ac-DEVD-CHO (*N*-acetyl-Asp-Glu-Val-Asp-aldehyde), Ac-LEHD-CHO (*N*-acetyl-Leu-Glu-His-Asp-aldehyde) or Z-IETD-fmk (benzyloxy-carbonyl-Ile-Glu-Thr-Asp-fluoromethylketone), inhibition of protein synthesis with cycloheximide or cell-cycle arrest with aphidicolin did not prevent cell death. Overexpression of Bcl-2, but not CrmA, efficiently prevented death in Jurkat cells. In all cell lines, death was always preceded by $\Delta\Psi_m$ loss and accompanied by the translocation of the protein apoptosis-inducing factor (AIF) from mitochondria to the nucleus. These results suggest that caspases are differentially involved in induction and execution of apoptosis depending on the leukaemic cell lineage. In any case, $\Delta\Psi_m$ loss marked the point of no return in apoptosis and may be caused by two different pathways, one caspase-dependent and the other caspase-independent. Execution of apoptosis was always performed after $\Delta\Psi_m$ loss by a caspase-9-triggered caspase cascade and the action of AIF.

Key words: AIF, apoptosis-inducing factor, 2-chloro-2′-deoxyadenosine, Mcl-1.

INTRODUCTION

The anti-neoplasic drug 2-chloro-2′-deoxyadenosine (2CdA, or cladribine) is a nucleoside analogue, resistant to deamination by adenosine deaminase and developed to selectively destroy lymphoid cells [1]. Although 2CdA is currently the drug of choice for hairy cell leukaemia [1] and it is also used for the treatment of B-cell chronic lymphocytic leukaemia (B-CLL) [2] and other lymphoproliferative syndromes [3], its mechanism of cytotoxicity remains unclear. It was initially assumed that, in proliferating cells, misincorporation of 2CdA into DNA could lead to growth arrest and cell death [4,5]. However, 2CdA is particularly efficient in the treatment of indolent lymphoid malignancies, suggesting the existence of an alternative mechanism for induction of apoptosis. Apoptosis induction in the case of B-CLL seems to be independent of p53 activity, since 2CdA induces apoptosis in cells with either wild-type or mutated p53 [6]. 2CdA is taken up by cells through a membrane transporter [7] and is converted in the cytoplasm to its triphosphate derivative, 2CdATP, by the sequential action of deoxycytidine kinase, AMP kinase and nucleoside diphosphate kinase [8]. It was suggested that 2CdATP could behave as a strong agonist of dATP (2′-deoxyribosyl-adenosine 5′-triphosphate) and activate Apaf-1 in the presence of low cytosolic concentrations of cytochrome *c* [9]. However, the relative potency of different halonucleotide analogues to bind and activate Apaf-1 did not correlate with their ability to induce apoptosis *in vivo* [10]. It has been also proposed that 2CdA may induce activation of the Fas/FasL system [11], but the relevance of the Fas pathway to 2CdA-induced apoptosis remains to be proved. Little is known about the implication of caspases and proteins of the Bcl-2 superfamily in the apoptosis induced by 2CdA. In B-CLL cells treated with fludarabine, a related nucleoside analogue, the general caspase inhibitor Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) influences the mode but not the extent of cell death [12]. On the other hand, the intracellular levels of Mcl-1, an anti-apoptotic protein of the Bcl-2 family, seem to correlate with the *in vivo* clinical response to fludarabine in some B-CLL patients [13,14].

In the present work we have studied the role of caspases and mitochondria in the apoptotic mechanism induced by 2CdA in several representative cell lines of human leukaemia and lymphoma. Results indicate that caspases are critically implicated in the induction phase of apoptosis in some, but not all, cell lines. In any case, mitochondrial transmembrane potential ($\Delta\Psi_m$) loss preceded the execution phase of cell death, which was mediated by activated caspases and the apoptosis-inducing factor (AIF) protein released from mitochondria.

EXPERIMENTAL

Materials

2CdA was kindly provided by Janssen-Cilag (Madrid, Spain). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 3-[4,5-di-

Abbreviations used: 2CdA, 2-chloro-2′-deoxyadenosine or cladribine; 2CdATP, triphosphate derivative of 2CdA; AIF, apoptosis-inducing factor; $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′-dihexyloxa-carbocyanine iodide; PS, phosphatidylserine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-IETD-fmk, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-LEHD-CHO, *N*-acetyl-Leu-Glu-His-Asp-aldehyde; B-CLL, B-cell chronic lymphocytic leukaemia; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PPDA, *p*-phenylenediamine; PE, phycoerythrin.

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methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and aphidicolin were from Sigma (Madrid, Spain). Mouse monoclonal anti-human caspase-3 antibody (clone 19), which recognizes the 32 kDa proenzyme, was from BDTransduction Laboratories (Madrid, Spain). Rabbit polyclonal anti-human active caspase-3, which recognizes the p17 subunit, was from BDPharmingen (Madrid, Spain). Rabbit polyclonal anti-human Mcl-1 was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit polyclonal anti-human caspase-9 (catalogue no. 68086E), which recognizes both the 46/48 kDa proenzyme and the 35/37 kDa subunit of the active form, was from BDPharmingen. Monoclonal anti-lamin B_1 (clone 101-B7) was from Calbiochem (Madrid, Spain) and monoclonal anti-β-actin (clone AC15) was from Sigma. Rabbit polyclonal anti-AIF antibody was kindly provided by Dr Santos Susin and Dr Guido Kroemer (CNRS, Villejuif, France). Z-VAD-fmk, Ac-DEVD-CHO (*N*-acetyl-Asp-Glu-Val-Asp aldehyde) and Ac-LEHD-CHO (*N*-acetyl-Leu-Glu-His-Asp-aldehyde) were from Bachem (Bubendorf, Switzerland). Z-IETD-fmk (benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone) was from Enzyme Systems Products (Dublin, CA, U.S.A.). Phycoerythrin (PE)-labelled annexin V was from Caltag (Barcelona, Spain) and 3,3′-dihexyloxa-carbocyanine iodide [$DiOC_6(3)$] was from Molecular Probes (Leiden, The Netherlands).

Cell proliferation and toxicity assays

The human T-cell leukaemia Jurkat (E6.1, TIB-152) and the pre-B leukaemia JM1 (CRL-10423) cell lines were from the A.T.C.C. The promonocytic leukaemia U937 cell line was kindly provided by Dr Jeremy Brock (University of Glasgow, Glasgow, U.K.). All the cell lines were cultured routinely at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine and penicillin/streptomycin (hereafter referred to as complete medium), using standard cell-culture procedures. Jurkat cell lines stably overexpressing viral CrmA protein, CrmA/Jurkat (clone L), and murine Bcl-2 protein, Bcl-2/Jurkat (clone 4), as well as vector-transfected cells, were provided kindly by Dr Daniel Johnson (University of Pittsburgh, Pittsburgh, PA, U.S.A.) and generated as described in [15]. Transfected cell lines were maintained in complete medium containing 1 mM Hepes and 0.5 mg/ml G418 (Life Technologies, Barcelona, Spain).

In proliferation assays, cells in the logarithmic phase of growth [$(3–7.5) \times 10^5$ cells/ml] were treated in flat-bottomed 96-well plates (100 μl/well) with different concentrations of 2CdA (0.1–15 μM) in complete medium for different times, as indicated. For apoptosis-inhibition assays, cells were incubated with one of the following inhibitors: 600 μM Ac-DEVD-CHO, 600 μM Ac-LEHD-CHO or 120 μM Z-IETD-fmk for 3 h, or for 1 h with 100 μM Z-VAD-fmk prior to the addition of 2CdA. Control cultures were treated with the appropriate amount of DMSO (0.1%, final concentration), used as a solvent for peptide inhibitors. In other experiments, the effect of blocking DNA or protein synthesis on apoptosis was evaluated. Cells were arrested in the G_1/S phases by a 10 h pretreatment with 2 μM aphidicolin, a specific inhibitor of DNA polymerases α and δ [16], prior to the addition of drug. Cycloheximide (0.25–0.5 μg/ml) was added to the cultures 1 h before 2CdA addition. This concentration of inhibitors did not cause any significant cytotoxicity. Cell proliferation was determined by a modification of the MTT-reduction method of Mosmann, as described in [17], and viability by microscopical evaluation of Trypan Blue-stained cells. In addition to stained cells, cells exhibiting a blebbing morphology, defined by the appearance of distinct protrusions of the plasma membrane and/or vacuolization, were scored as non-viable. Chromatin condensation and nuclear fragmentation during apoptosis were analysed by labelling cell nuclei with *p*-phenylenediamine (PPDA) in oxidized glycerol and visualized by fluorescence microscopy as described previously [18].

Immunofluorescence and flow-cytometry analysis

Cells (1×10^6 in 0.5 ml) were seeded in complete medium with or without 2CdA (0.5–1 μM) and cultured for 16 h. At the end of incubation, cells were washed once in PBS and fixed with 4% paraformaldehyde for 15 min. Then, cells were centrifuged on to poly-L-lysine-coated glass coverslips. Coverslips were then washed once in PBS and briefly immersed in 0.1% saponin in PBS. The coverslips were placed on to a drop of a 1/100 dilution of rabbit anti-AIF antiserum and incubated at room temperature in a humidified chamber for 30 min. Coverslips were then washed twice with a saponin solution and incubated in the same way with a 1/100 dilution of a FITC-labelled anti-rabbit IgG antibody (Caltag). Finally, coverslips were washed sequentially with 0.1% saponin, PBS and distilled water, mounted on to glass slides over a drop of Mowiol (Calbiochem) and stored at 4 °C in the dark until observation. Preparations were observed using a Zeiss 310 confocal microscope, and analysed using LSM 3.95 software. Phosphatidylserine (PS) exposure during apoptosis was evaluated by annexin V-PE binding. Briefly, cells [$(2–2.5) \times 10^5$] were washed with PBS and incubated in a solution of 0.5 μg/ml annexin V-PE in binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$ and 10 mM Hepes/NaOH, pH 7.4), at room temperature for 30 min. Then cells were washed, resuspended in 1 ml of binding buffer and finally 5000 cells/sample were analysed in an Epics XL-MCL flow cytometer (Coulter, Madrid, Spain). The amount of hypodiploid cells was evaluated after extraction of degraded DNA with phosphate/citrate buffer and cell DNA staining with propidium iodide, as described in [19]. To evaluate $\Delta\Psi_m$, the cationic lipophilic fluorochrome $DiOC_6(3)$ was used [20]. Cells (1.5×10^5 in 200 μl complete medium) were incubated with 40 nM $DiOC_6(3)$ for 20 min at 37 °C, diluted with PBS to a final volume of 1 ml and analysed by flow cytometry. As a negative control, cells were treated in parallel samples with the protonophore uncoupling agent CCCP (50 μM).

Western-blot analysis

Caspase activation or levels of apoptosis-regulatory proteins were evaluated by Western-blot analysis of cell homogenates using specific antibodies. Cells were treated in complete medium with 2CdA for the times indicated, and cell viability determined by Trypan Blue staining. Cells were recovered by centrifugation, washed with PBS and lysed in the appropriate volume of lysis buffer (0.15 M NaCl, 1 mM EDTA, 30 mM NaF, 10 μg/ml leupeptin, 1 mM PMSF and 50 mM Tris/HCl, pH 7.6) containing 1% Triton X-100. Solubilized proteins from equal numbers of extracted cells [$(2–3) \times 10^6$ Trypan Blue-negative cells for each lane] were resolved by SDS/PAGE (12% gels) and transferred to nitrocellulose membranes as described previously [18]. Membranes were incubated with one of the following primary antibodies diluted in TBS-T (10 mM Tris/HCl, pH 8, 0.12 M NaCl, 0.1% Tween-20 and 0.05% sodium azide) containing 5% skimmed milk powder: 0.25 μg/ml anti-caspase-3, a 1/6000 dilution of anti-caspase-9, a 1/50 dilution of anti-lamin B_1, a 1/500 dilution of anti-Mcl-1, a 1/500 dilution of anti-Bcl-2 or a 1/500 dilution of anti-Bax. Then, membranes were washed with TBS-T and incubated with 0.2 μg/ml of the corresponding

alkaline phosphatase-labelled secondary antibody, immunoadsorbed with human serum proteins (Sigma). Western blots were revealed with the 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) substrate, as described in [21]. Control of protein loading was achieved by reblotting with anti-β-actin (1/10000).

RESULTS

Effect of 2CdA on cell proliferation and viability

In all cell lines tested, 2CdA caused cell death in a dose- and time-dependent way (Figure 1A), although the relative sensitivity of cell lines to 2CdA varied. Analysis of cell growth, by using the

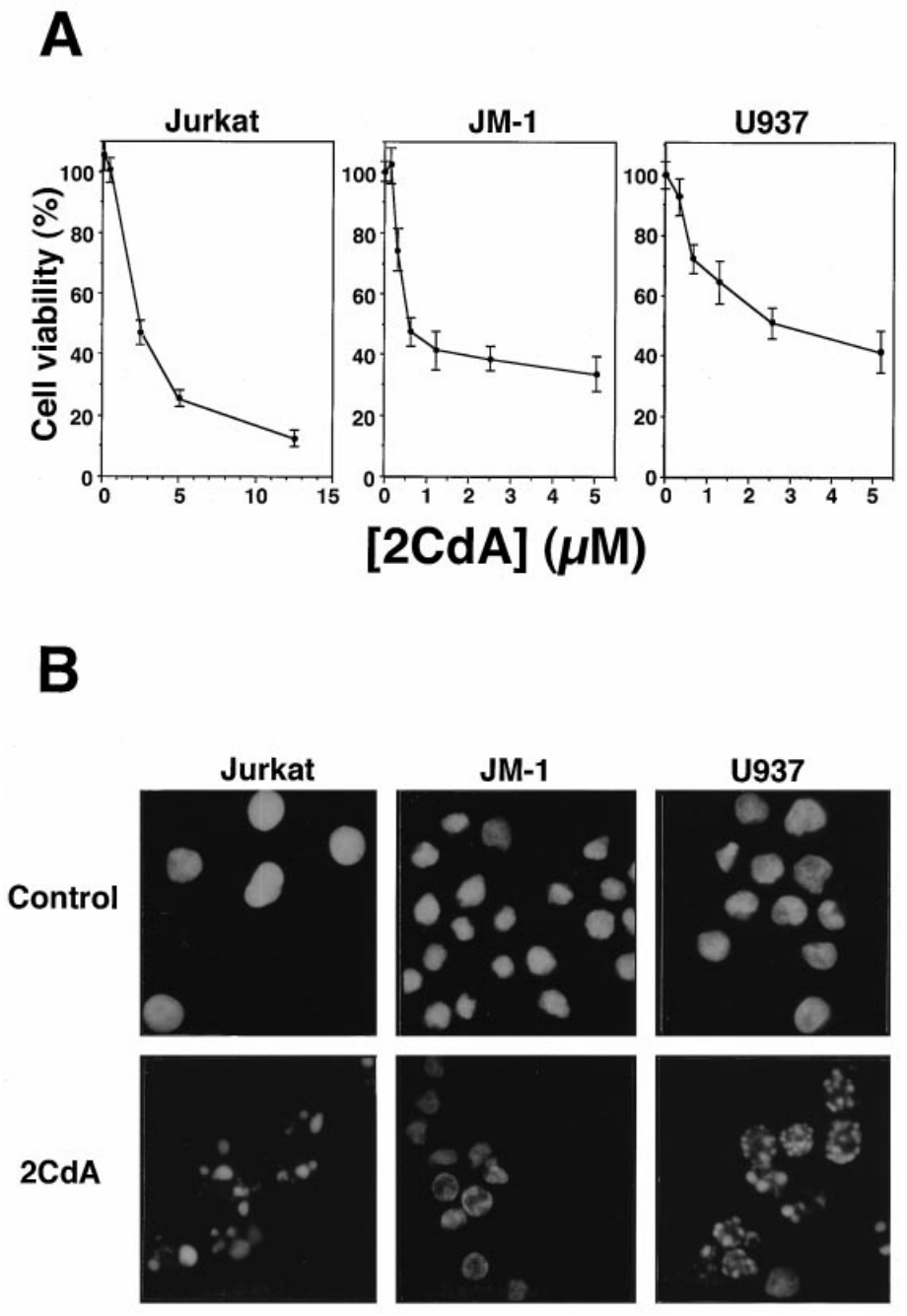

Figure 1 Effect of 2CdA on viability of human leukaemia cells

(**A**) Jurkat (5×10^5 cells/ml), JM1 (7.5×10^5 cells/ml) or U937 (3×10^5 cells/ml) cells were treated in 96-well plates (100 μl/well) in complete medium with different concentrations of 2CdA. Cell proliferation was estimated at 16 h (JM1, U937) or 48 h (Jurkat) by the MTT assay. Results are means $\pm$ S.D. from three or four individual determinations on two different experiments and are expressed as the percentage of cell viability relative to controls without drug. (**B**) Cells were treated with 5 μM (Jurkat), 0.5 μM (JM1) or 1 μM (U937) 2CdA for 18 h, stained with PPDA and photographed under epifluorescence illumination. Typical apoptotic chromatin condensation and nuclear fragmentation can be observed in Jurkat and U937 cells but not in JM1 cells. Magnification, $\times$ 280.

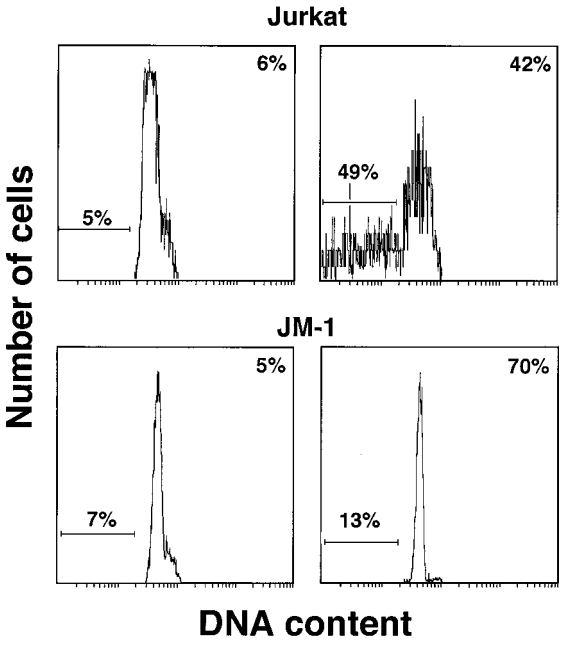

Figure 2 Treatment with 2CdA induces hypodiploidy in Jurkat but not in JM-1 cells

Jurkat (3×10^5 cells/ml) or JM1 (5×10^5 cells/ml) cells were left untreated (left-hand panels) or incubated with 5 μM or 0.5 μM 2CdA in complete medium for 18 h (right-hand panels). Then cells were stained with propidium iodide and fluorescence analysed by flow cytometry. The percentages of sub-G_1 hypodiploid cells (bars) and dead cells (upper right corner) are indicated in each case. Cell death was determined in parallel samples by Trypan Blue staining.

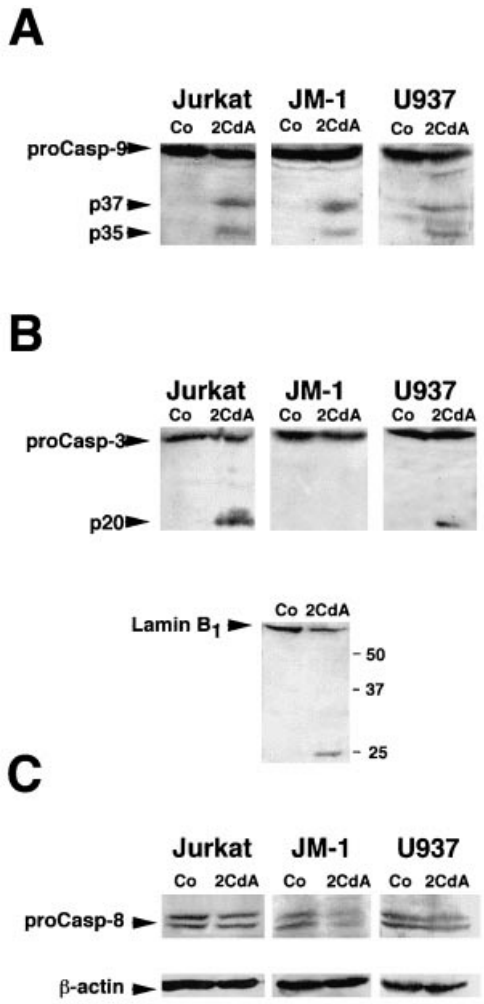

Figure 3 Caspase activation induced by 2CdA

Caspase activation was analysed by Western blotting using specific antibodies for (**A**) caspase-9, (**B**) caspase-3 and (**C**) caspase-8. Activation of caspase-6 was confirmed by analysis of lamin B_1 cleavage (**B**). Immunoblots were reprobed with anti-β-actin antibody as a control for equal loading. Co, control.

MTT-reduction method, indicated that pre-B JM1 cells were the most sensitive to 2CdA, with an IC_{50} of 0.5 μM at 16 h, followed by U937 cells (IC_{50}, 2.5μM at 16 h) and Jurkat (IC_{50}, 2.5 μM at 48 h). Microscopical evaluation of 2CdA-treated JM1 cells revealed that dead cells offered some morphological characteristics of apoptosis, namely cell shrinking and an atypical marginal condensation of chromatin, but not nuclear fragmentation. Jurkat and U937 cells displayed additional morphological features of apoptosis, such as typical chromatin condensation, nuclear fragmentation, membrane blebbing and formation of apoptotic bodies (Figure 1B). The lack of nuclear fragmentation in JM1 cells was further confirmed by evaluating the amount of hypodiploid cells after 2CdA treatment by nuclear propidium iodide staining and flow cytometry. As shown in Figure 2, Jurkat, but not JM1 cells, showed a significant degree of nuclear fragmentation in spite of the great amount of apoptotic cells. Also, the DNA extracted form JM1 cells treated with 2CdA lacked the typical oligonucleosomal ladder pattern (results not shown).

Analysis of caspase activation induced by 2CdA

Caspase activation during 2CdA-induced apoptosis of JM1, Jurkat and U937 cells was analysed by Western blotting. 2CdA induced activation of caspase-9 in all cell lines examined. The intensity of a protein doublet at 35/37 kDa, corresponding to the large subunit of the processed enzyme, increased in cells treated with 2CdA (Figure 3A). 2CdA also induced a significant caspase-3 activation in Jurkat and U937 cells, as shown by reduction in the intensity of the proenzyme and the appearance of the 19 kDa band of the active subunit (Figure 3B). This band was also observed in JM1 cells treated with 2CdA, although it was weaker than in Jurkat cells. Nonetheless, activation of caspase-3 in JM1 cells was enough to activate caspase-6, as estimated by analysis of cleavage of its specific substrate, lamin B_1 [22] (Figure 3B). Caspase-8 was also activated upon 2CdA treatment in JM1 and U937 cells and, to a lesser extent, in Jurkat cells (Figure 3C).

Effect of caspase inhibitors on 2CdA-induced apoptosis

It has been reported that at concentrations greater than 1 nM $DiOC_6(3)$ is not only sensitive to changes in $\Delta\Psi_m$, but also in plasma-membrane potential [23]. Control experiments revealed that the percentage of cells displaying high or low $\Delta\Psi_m$ values under various experimental conditions (either treated or untreated with 2CdA in the presence or absence of Z-VAD-fmk) were similar at $DiOC_6(3)$ concentrations ranging between 1 and 40 nM. Moreover, the amplitude of loss of $\Delta\Psi_m$ in response to the protonophore CCCP was similar in all cases. The mean fluorescence intensity and the fluorescence gap between both peaks increased with increasing concentrations of $DiOC_6(3)$.

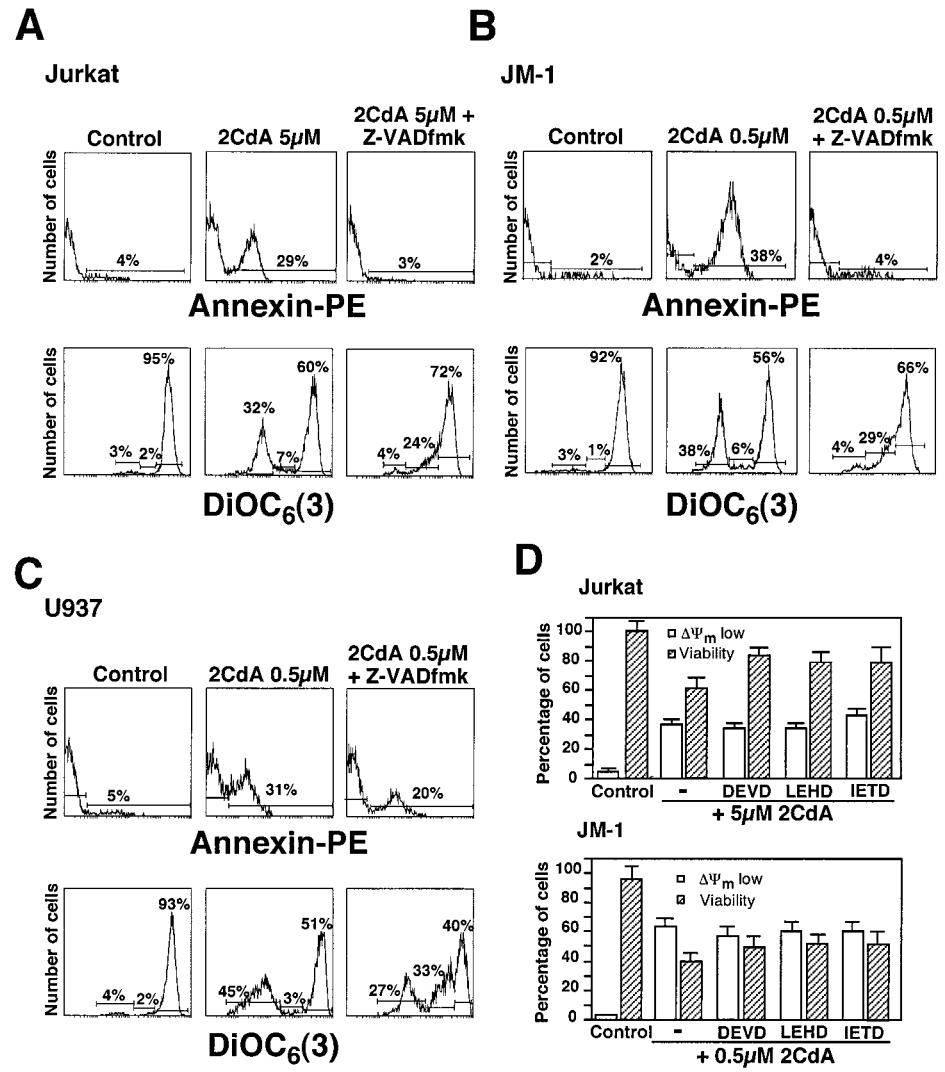

Figure 4 Effect of peptide caspase inhibitors on 2CdA-induced apoptosis

(**A**) Jurkat, (**B**) JM1 and (**C**) U937 cells (3×10^5cells/ml) were incubated for 18 h with 2CdA in the presence or absence of 100 μM Z-VAD-fmk and $\Delta\Psi_m$ and PS exposure analysed by flow cytometry by using the fluorescent probe $DiOC_6(3)$ and annexin V-PE, respectively. Numbers refer to the percentages of cells in the different regions of flow-cytometric profiles. The *x*-axes refer to the log of fluorescence intensity. The experiments shown are representative of five different experiments for each cell line. (**D**) Jurkat or JM1 cells were cultured for 18 h in complete medium containing 2CdA (5 μM and 0.5 μM, respectively) in the absence (control) or presence of 600 μM Ac-DEVD-CHO, 600 μM Ac-LEHD-CHO or 120 μM Z-IETD-fmk. Cell viability was assessed by flow cytometry using annexin V-PE and $\Delta\Psi_m$ with $DiOC_6(3)$. Results (means $\pm$ S.D.) are representative of three different experiments for each cell line.

Since 40 nM $DiOC_6(3)$ provided the highest sensitivity, while maintaining specificity, this concentration was used in all experiments. Fluorescence microscopy analysis revealed that $DiOC_6(3)$ staining followed a punctate cytoplasmic pattern, typical of a mitochondrial localization, with no visible contribution of the plasma membrane. Treatment with CCCP caused the complete loss of $DiOC_6(3)$ staining, whereas incubation in the presence of 0.15 M KCl, which abolishes plasma-membrane potential, did not affect the fluorescence labelling (results not shown). This has been also demonstrated in a recent flow-cytometric study [24].

Treatment of Jurkat, JM1 and U937 cells with 2CdA induced PS exposure and loss of $\Delta\Psi_m$ (Figures 4A–4C). In Jurkat and JM1 cells, co-treatment with the general caspase inhibitor Z-VAD-fmk completely prevented development of apoptotic morphology and PS exposure induced by 2CdA at concentrations that caused 30–50 % mortality (Figures 4A and 4B). By contrast, PS exposure induced by 2CdA in U937 cells was only slightly inhibited by Z-VAD-fmk (Figure 4C), although release of apoptotic bodies decreased in the presence of this inhibitor. Inhibition by Z-VAD-fmk of $\Delta\Psi_m$ loss caused by 2CdA in Jurkat and JM1 cells was not complete. A significant portion of cells showed an intermediate $\Delta\Psi_m$ ($\Delta\Psi_m{}^{int}$) when compared with untreated cells ($\Delta\Psi_m{}^{high}$) and cells treated with 2CdA alone ($\Delta\Psi_m{}^{low}$; Figures 4A and 4B). In U937 cells, Z-VAD-fmk did not prevent $\Delta\Psi_m$ loss, although this process seemed to be retarded since around 30 % of cells showed an $\Delta\Psi_m{}^{int}$ (Figure 4C). The selective inhibitors Ac-DEVD-CHO (inhibitor of caspases 3, 7 and 8), Z-IETD-fmk (caspases 6, 8, 9 and 10) and Ac-LEHD-CHO (caspase-9) failed to prevent toxicity of 2CdA in JM1 or Jurkat cells (Figure 4D), although we observed a slight increase in viability of Jurkat cells treated with 2CdA in the presence of these inhibitors (Figure 4D). None of the selective inhibitors abrogated the loss of $\Delta\Psi_m$ induced by 2CdA in Jurkat, JM1 and U937 cells. Nevertheless, nuclear condensation and fragmen-

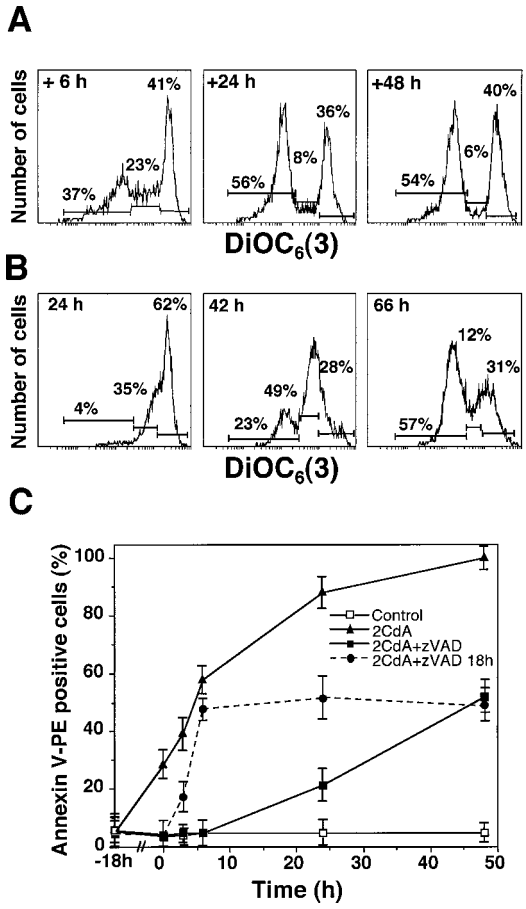

Figure 5 Effect of Z-VAD-fmk on 2CdA-induced $\Delta\Psi_m$ loss and apoptosis

(**A**) Jurkat cells (3×10^5 cells/ml) were incubated for 18 h with 5 μM 2CdA and 100 μM Z-VAD-fmk, washed, resuspended in fresh complete medium and cultured at 37 °C. At different time points (indicated as insets), $\Delta\Psi_m$ was assessed by flow cytometry with $DiOC_6(3)$. (**B**) Jurkat cells were treated with 5 μM 2CdA and 100 μM Z-VAD-fmk and $\Delta\Psi_m$ was assessed at different time points as indicated. In (**A**) and (**B**) numbers refer to the percentages of cells in the different regions of flow-cytometric profiles. The *x*-axes refer to the log of fluorescence intensity. (**C**) Evaluation of PS translocation, by annexin V-PE binding, of Jurkat cells treated as in (**A**) and (**B**), as well as in untreated cells (Control) or cells treated with 5 μM 2CdA alone. Results are the means ± S.D. from three to five individual determinations in two different experiments.

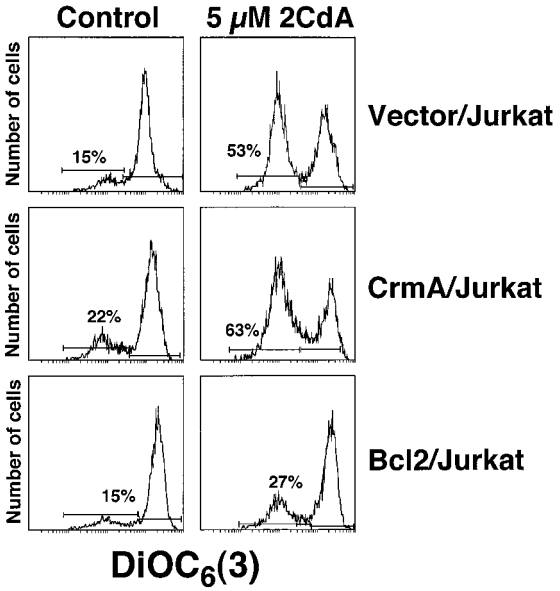

Figure 6 Effect of CrmA or Bcl-2 overexpression on 2CdA-induced apoptosis

Jurkat cells overexpressing CrmA, Bcl-2 or vector-transfected cells were incubated for 24 h in medium containing 5 μM 2CdA. $\Delta\Psi_m$ was assessed by flow cytometry with $DiOC_6(3)$. Results are representative of three different experiments. Numbers refer to the percentages of cells in the different regions of flow-cytometric profiles. The *x*-axes refer to the log of fluorescence intensity.

tation was inhibited in Jurkat cells treated with 2CdA in the presence of Ac-DEVD-CHO. In this case, a high percentage of Jurkat cells exhibited nuclei showing peripheral chromatin condensation, as shown by PPDA staining (results not shown).

We also evaluated whether cells treated with 2CdA in the presence of Z-VAD-fmk and displaying an intermediate $\Delta\Psi_m$ were committed to death (Figure 5). Jurkat and JM1 cells were incubated with 2CdA (5 and 0.5 μM, respectively) and 100 μM Z-VAD-fmk for 18 h, washed and resuspended in fresh complete medium. At different time points after 2CdA and Z-VAD-fmk removal, cell morphology, viability, $\Delta\Psi_m$ and PS exposure were analysed. Cells incubated with 2CdA in the presence of Z-VAD-fmk for 18 h began to die when both agents were withdrawn. This process was rapid and annexin V-positive cells could already be detected 2 h after washing (Figure 5C). The percentage of annexin V-positive Jurkat cells was around 45 % after 6 h and did not increase for the next 48 h. During this period, there was a comparable increase in the percentage of cells exhibiting a low $\Delta\Psi_m$ (Figure 5A). The number of dead cells showing an apoptotic phenotype was also scored by light microscopy at different times after washing. As shown in Figure 5(C), the percentage of apoptotic Jurkat cells increased quickly after the removal of Z-VAD-fmk and 2CdA, reaching approx. 40 % of total cells, a figure similar to the percentage of cells with an intermediate $\Delta\Psi_m$ at the moment of 2CdA/Z-VAD-fmk removal. The proportion of non-viable cells did not increase after this initial period but apoptotic cells became permeable to Trypan Blue in the next 24 h. In other experiments, cells were incubated for long periods with 2CdA in the continuous presence of Z-VAD-fmk. In this case, cell viability was preserved for the first $\approx$ 40 h and began to decrease thereafter (Figures 5B and 5C). This decrease was even more rapid for JM1 cells incubated with 0.5 μM 2CdA and by 72 h viability was almost entirely lost (results not shown). Therefore, the cell subpopulation displaying an intermediate $\Delta\Psi_m$ after 2CdA treatment in the presence of Z-VAD-fmk was effectively committed to die.

On the other hand, anti-Fas blocking antibodies were unable to block 2CdA-induced apoptosis in Jurkat and U937 cells, whereas JM1 cells were resistant to Fas-induced apoptosis (results not shown). These results, together with the lack of inhibition of cell death by Ac-DEVD-CHO, ruled out the possibility that apoptosis induced by 2CdA was mediated by the Fas/FasL system.

Effect of CrmA or Bcl-2 overexpression on apoptosis

To further delineate the role of mitochondria in 2CdA-induced apoptosis, Jurkat cells overexpressing CrmA (a specific inhibitor of caspase-8) or Bcl-2 (a regulator of mitochondrial pore opening) were treated with 2CdA. Overexpression of Bcl-2, but not CrmA, prevented loss of $\Delta\Psi_m$, prevented PS exposure (Figure 6) and prevented development of apoptotic morphology induced by

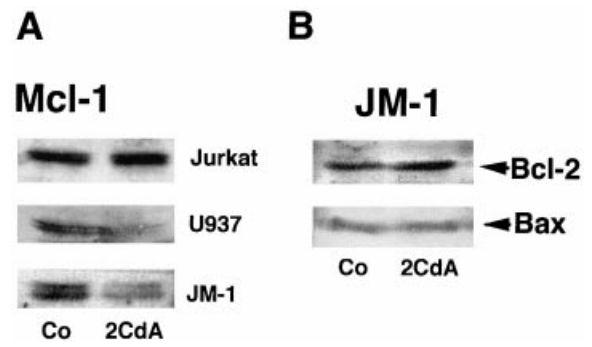

Figure 7 Effect of 2CdA on Mcl-1 levels

(**A**) Cells (3×10^5 cells/ml) were incubated in complete medium with or without 5 μM 2CdA (Jurkat) or 0.5 μM 2CdA (JM1, U937) for 18 h. Cell homogenates were prepared as described in the Experimental section and Mcl-1 levels analysed by Western blotting. (**B**) JM-1 cells were treated as in (**A**) and the levels of Bcl-2 and Bax analysed by Western blotting. Co, control.

2CdA (results not shown). In addition, caspases 9 and 3, and to a lesser extent caspase-8, were activated in CrmA/Jurkat cells upon 2CdA treatment, indicating that caspase-8 was activated downstream of caspase-3. In CrmA/Jurkat cells triggered with anti-Fas, a pathway in which caspase-8 is the apical caspase, no caspase activation and apoptosis were observed (results not shown and [15,21]), according to the known inhibition of caspase-8 by CrmA. No activation of caspases 9 and 3 was observed in Bcl-2/Jurkat cells treated with 2CdA (results not shown), according to the lack of cell death. These results indicate that 2CdA induces cell death through the mitochondrial pathway of apoptosis.

Effect of cycloheximide and aphidicolin on 2CdA toxicity

To analyse the dependence of 2CdA toxicity on *de novo* protein synthesis, Jurkat, JM1 and U937 cells were incubated with this drug in the presence of the protein-synthesis inhibitor cycloheximide. In all cases, there was no significant inhibition of $\Delta\Psi_m$ or PS exposure, indicating that protein synthesis is not necessary for 2CdA-induced apoptosis in these cell lines. To evaluate whether incorporation of 2CdA into DNA was necessary for the triggering of apoptosis, we performed experiments using the DNA polymerase inhibitor aphidicolin. After incubation with aphidicolin for 10 h, most cells were blocked at the G_1/S transition, as determined by flow cytometry (results not shown). This caused no significant inhibition of toxicity induced by 2CdA in JM1 and U937 cells, indicating that, in these cell lines, incorporation of 2CdA into DNA is not essential for the initiation of cell death. Aphidicolin treatment significantly reduced 2CdA toxicity in Jurkat cells at 16 h (9 % of cell death in treated versus 38 % in untreated cells), but not at 40 h (70 % cell death in aphidicolin-treated versus 93 % in untreated cells). This suggests that incorporation of 2CdA into DNA of Jurkat cells may contribute to the early induction of death signals.

Effect of 2CdA on levels of Bcl-2-family proteins

It has been reported that levels of Mcl-1, an anti-apoptotic member of the Bcl-2 family [25], inversely correlate with the response rates to fludarabine in patients with B-CLL [13,14]. We have analysed the changes induced by 2CdA treatment in the levels of Mcl-1, Bcl-2 and Bax proteins. Mcl-1 levels decreased in JM1 and U937, but not in Jurkat cells treated with 2CdA

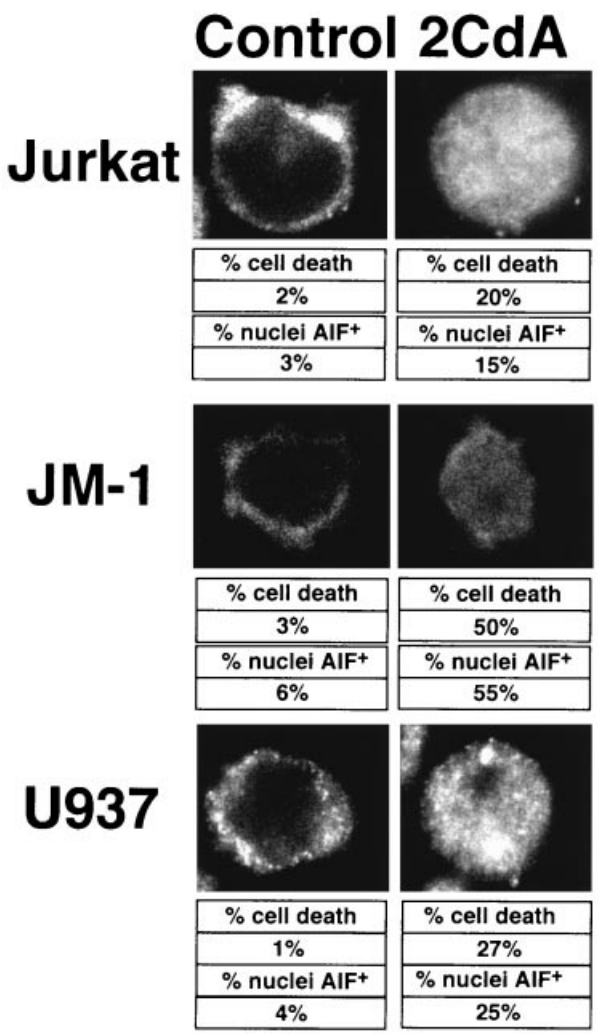

Figure 8 Translocation of AIF from mitochondria to nucleus induced by 2CdA

Immunofluorescence localization of AIF in Jurkat, JM1 and U937 cells. Cells (1×10^6 in 2 ml) were incubated for 16 h in complete medium (Control) or medium containing 5, 0.5 and 1 μM 2CdA, respectively. After treatment, cells were fixed and sequentially labelled with anti-AIF and anti-rabbit IgG FITC and analysed by confocal microscopy. Magnification, $\times 950$.

(Figure 7A). No significant changes in the levels of Bax and Bcl-2 proteins in JM1 cells upon 2CdA treatment were observed (Figure 7B). Jurkat cells, either treated or untreated with 2CdA, expressed barely detectable levels of Bax and Bcl-2 proteins.

2CdA induces nuclear traslocation of AIF

AIF is a mitochondrial protein that, upon $\Delta\Psi_m$ loss, is released into the cytoplasm and imported into the nucleus [26]. Nuclear AIF induces, through a caspase-independent mechanism, peripheral and 'dot' chromatin condensation and large-scale DNA fragmentation [26]. This type of nuclear apoptotic morphology can be seen in JM1 cells treated with 2CdA (Figure 1). We analysed by immunofluorescence the intracellular localization of AIF before and after the treatment of JM1 cells with 2CdA. Confocal microscopy revealed that untreated cells showed a punctated, mainly perinuclear, localization of AIF (Figure 8A). AIF labelling co-localized with fluorescence distribution of the

specific mitochondrial probe $DiOC_6(3)$ (results not shown). After incubation with 2CdA, immunostaining revealed a diffuse fluorescence pattern and a change in the distribution of AIF, which was mainly localized inside the nucleus (Figure 8) in a high percentage of cells. Similar results were obtained with Jurkat and U937 cells (Figure 8), but this labelling was more clearly defined in cells with a still unfragmented nucleus. AIF translocation was also observed in Jurkat and U937 cells treated with 2CdA in the presence of the caspase-3 inhibitor Ac-DEVD-CHO (results not shown), which prevented nuclear fragmentation, indicating that AIF translocation was independent of caspase-3. In these cells, a pattern of nuclear condensation similar to that found in JM1 cells treated with 2CdA (see Figure 1B) could be observed.

DISCUSSION

2CdA is a cytotoxic drug designed to selectively destroy lymphoid cells and which is currently used for the treatment of B-cell neoplasias [1,2]. In sensitive cells, 2CdA induces apoptosis through a mechanism that is still incompletely understood. Recent reports have shown that caspases are implicated in apoptosis induced by 2CdA [9,11,27] and by fludarabine [14,28], a related nucleoside analogue, in B-CLL cells. However, the precise role of caspases in cell death elicited by nucleoside analogues is not fully delineated. It has been suggested recently that caspases are implicated in the execution of, not in the commitment to, apoptosis in B-CLL cells [12], but it is not known if this is also applicable to other lymphoid cells. In fact, another report proposed that 2CdA-induced apoptosis in Molt-4 leukaemia cells was mediated by the Fas/FasL system [11] and fully inhibited by caspase inhibitors. In the present work we have analysed the role of caspases in apoptosis induced by 2CdA in different leukaemic cells, with the aim of providing a rational basis for its use in *in vivo* chemotherapy.

According to current knowledge, cytotoxic drugs cause apoptosis mainly using the so-called 'mitochondrial pathway'. In this pathway, apoptosis is induced by an intrinsically generated death signal which arrives at mitochondria causing loss of $\Delta\Psi_m$ and the release of cytochrome *c* and AIF into the cytoplasm. Cytochrome *c* binds to the adaptor Apaf-1 and the complex recruits and activates caspase-9, which initiates a caspase cascade responsible for the hydrolysis of key cytoplasmic proteins [29]. On the other hand, AIF migrates to the nucleus and induces high-molecular-mass DNA fragmentation and marginal chromatin condensation [26]. The combined action of caspases and AIF causes the apoptotic phenotype (cell shrinking, blebbing, nuclear condensation and fragmentation) and cell death. In most cases, loss of $\Delta\Psi_m$ marks the point of no return between the induction and the execution phases of apoptosis [30]. While the involvement of caspases in the execution of drug-induced apoptosis is firmly documented, their participation in the induction phase is not well defined. In some leukaemic cells, caspases may be involved in the onset of cell death and so co-treatment with Z-VAD-fmk blocks the development of apoptotic phenotype and cell death [31–33]. However, in other cell types or with other drugs, caspases seem only to be involved in the execution of apoptosis, and Z-VAD-fmk does not prevent death [34,35].

Our present results indicate that 2CdA can induce apoptosis in leukaemic cells from different lineages, which is manifested by $\Delta\Psi_m$ loss, caspase activation, AIF translocation, PS exposure and chromatin condensation. All the molecular machinery needed for these processes was already present in the cells, since inhibition of *de novo* protein synthesis with cycloheximide did not significantly alter the rate or extent of death. However, caspases seem to play distinct roles in apoptosis depending on the cell type. In all leukaemic cell lines, inhibitors of caspases 3 and 7 (Ac-DEVD-CHO) or caspases 8 and 9 (Z-IETD-fmk and Ac-LEHD-CHO) mitigated PS exposure and prevented chromatin condensation and cellular fragmentation but did not block $\Delta\Psi_m$ loss and cell death. Under the same conditions, co-treatment with the pan-caspase inhibitor Z-VAD-fmk inhibited all the morphological features of apoptosis, as well as PS translocation, $\Delta\Psi_m$ loss and cell death in Jurkat and JM1, but not U937, cells. These results suggest that a Z-VAD-sensitive caspase, distinct from caspases 3, 7, 8 and 9, causes $\Delta\Psi_m$ loss and commitment to death in Jurkat and JM1 cells. In the case of U937 cells, caspases are not crucial for this step, which would be mediated by other factor(s). In any case, 2CdA-induced apoptosis is not mediated by the Fas/FasL system, since co-incubation with either blocking anti-Fas antibodies or Ac-DEVD-CHO, any of which efficiently block Fas-induced apoptosis [18,36], did not prevent cell death. Moreover, it has been also demonstrated that apoptosis induced by fludarabine or 2CdA in CEM T-cells is independent of the Fas/FasL system [37,38].

Results also show that 2CdA may provoke a reduction in $\Delta\Psi_m$ in the absence of caspase activity. As mentioned, complete $\Delta\Psi_m$ loss occurred in the presence of Z-VAD-fmk in U937 cells. In JM1 cells, and to a lesser extent in Jurkat cells, a significant portion showed an intermediate $\Delta\Psi_m$ when incubated with 2CdA in the presence of Z-VAD-fmk. These cells, although still viable, are committed to death, since withdrawal of Z-VAD-fmk and 2CdA causes a rapid augmentation of the percentage of apoptotic cells. This percentage was similar to the percentage of cells exhibiting an intermediate $\Delta\Psi_m$ at the moment of drug removal, which suggests that this cell subpopulation has gone beyond the point of no return in apoptosis. The percentage of cells still displaying a high $\Delta\Psi_m$ remained roughly constant after the removal of 2CdA from medium and maintained their viability, at least for the next 48 h. In the continuous presence of 2CdA, complete caspase inhibition with Z-VAD-fmk did not indefinitely preserve cell viability and $\Delta\Psi_m$ was lost and cells began to die after $\approx$ 40 h.

According to these and previous [33,34,39] results, loss of $\Delta\Psi_m$ during apoptosis induced by 2CdA in JM1 and Jurkat cells might occur in two steps. First, a non-caspase factor, acting on mitochondria, would cause a partial reduction in $\Delta\Psi_m$, enough to signal cell commitment to death. Next, perhaps after the release of a small amount of cytochrome *c* [39], a slight activation of caspases, among them 9 and 3, would in turn enhance $\Delta\Psi_m$ loss [40], causing the release of AIF [41] and further caspase activation. Thus caspases would engage in a feedback amplification loop that would accelerate the apoptotic process [39]. However, the key caspase responsible for the complete $\Delta\Psi_m$ loss and apoptosis induction in JM1 and Jurkat cells remains to be identified. This caspase must be different from caspases 3, 7, 8 and 9 since complete $\Delta\Psi_m$ loss still occurs under conditions of complete inhibition of these caspases, i.e. in the presence of Ac-DEVD-CHO and Ac-LEHD-CHO. In the presence of Z-VAD-fmk, all caspases are inhibited and this causes a significant delay (around 40 h in our experimental conditions) in the execution of cell death. However, cells eventually die because of the progressive loss of $\Delta\Psi_m$ and the release of AIF. In U937 cells, the non-caspase factor is probably more efficient in causing loss of $\Delta\Psi_m$ and so inhibition of caspases only slightly retarded death although it preserved cell morphology.

Our results show that AIF is released from mitochondria and translocated to the nucleus in JM1 and Jurkat cells treated with 2CdA. In JM1 cells, the typical aspect of chromatin condensation and nuclear fragmentation is not observed, despite the activation

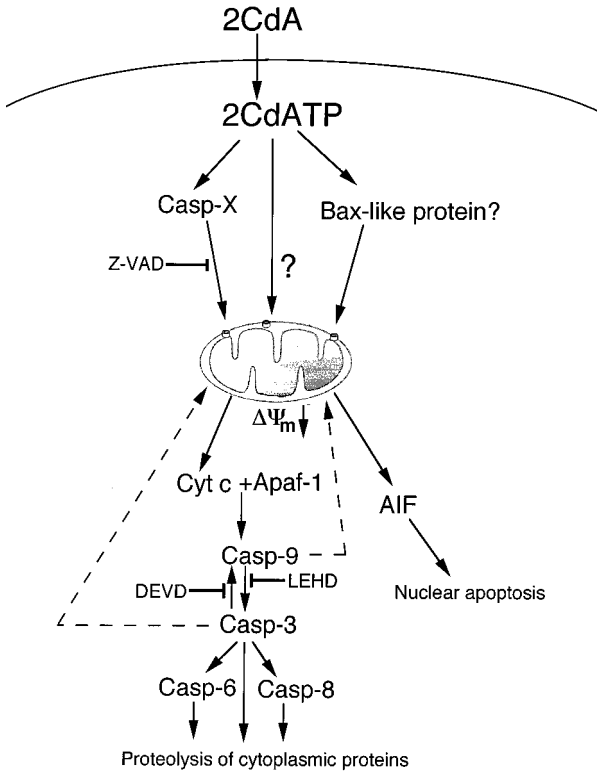

Figure 9 Hypothetical scheme of 2CdA-induced apoptosis

2CdA is incorporated by cells and converted to its triphosphate derivative (2CdATP), which might induce mitochondrial dysfunction in three different ways: (i) through the activation of an unidentified Z-VAD-sensitive caspase, (ii) by causing the translocation to mitochondria of a Bax-like protein or (iii) by directly inducing mitochondrial permeability transition. Pathway (i) would be predominant in Jurkat and JM1 cells, whereas (ii) would be preponderant in U937 cells. In all cases, the net result is loss of $\Delta\Psi_m$ and the release of cytochrome *c* and AIF. Cytochrome *c* binds to Apaf-1 and the complex activates caspase-9, triggering a caspase cascade that cleaves key cytoplasmic proteins. AIF is translocated to the nucleus and contributes to chromatin condensation and fragmentation.

of caspases. It is likely that these cells carry a defect in the pathway leading to oligonucleosomal DNA fragmentation, downstream of caspase-3, since the same pattern of chromatin margination was observed with other chemically unrelated drugs (I. Marzo and J. Naval, unpublished work). AIF could be the responsible for the peripheral chromatin condensation observed after $\Delta\Psi_m$ loss in JM1 cells. This lack of classical nuclear apoptotic morphology, in spite of caspase-3 activation, has also been found in the B-lymphoma Raji [42]. In the case of Jurkat and U937 cells, the AIF-induced nuclear morphology is masked by the action of caspase-activated DNase (CAD), which causes further chromatin condensation and nuclear fragmentation [43]. Accordingly, an AIF-induced morphology can be observed in Jurkat and U937 cells treated with 2CdA in the presence of Ac-DEVD-CHO.

On the other hand, the non-caspase factor responsible for the commitment to death remains to be identified, but a proapoptotic member of the Bcl-2 superfamily is an attractive candidate. We have found that 2CdA induced a decrease in the expression of anti-apoptotic Mcl-1 in JM1 (B-cells), but not in Jurkat (T-cells), congruent with a possible role of Mcl-1 in apoptosis induced by 2CdA in B-cells [13,14]. Levels of Bax and Bcl-2 proteins did not significantly change with 2CdA treatment, as previously found with fludarabine [44–46]. However, Bcl-2 overexpression prevented 2CdA-induced apoptosis in Jurkat cells. A possible role of Bid can be ruled out, since Bid requires prior proteolytic activation by caspase-8 [47], and inhibition of caspase-8 with Ac-IETD-CHO did not prevent apoptosis. A direct effect of 2CdATP on mitochondria has been also postulated [27] but this does not agree with the differences in apoptosis induction observed between Jurkat, JM1 and U937 cells. Anyway, the incorporation of 2CdA into DNA does not seem to be a prerequisite for toxicity, since cell-cycle arrest in the G_1/S phases with aphidicolin had no effect on apoptosis in JM1 and U937 cells and only delayed death of Jurkat cells. This is in contrast to the toxicity of fludarabine in Jurkat and other proliferating cells which is blocked by aphidicolin (results not shown and [48]). A hypothetical scheme describing the possible role of mitochondria, caspases and AIF in 2CdA-induced apoptosis is shown in Figure 9.

In summary, caspases and AIF are the executioners of 2CdA-induced apoptosis in all leukaemic cells tested. The main determinant of $\Delta\Psi_m$ loss and commitment to death is an unidentified Z-VAD-sensitive caspase in Jurkat and JM1 cells and a non-caspase factor in U937 cells. This non-caspase factor would also eventually lead to cell death in Jurkat and JM1 cells at longer times if caspase activity is blocked. The characterization of these unidentified apoptotic factors may be important in the elucidation of the mechanism of 2CdA-induced apoptosis and in the understanding of the process of drug resistance.

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