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ORIGINAL ARTICLE

The role of cordycepin in cancer treatment via induction or inhibition of apoptosis: implication of polyadenylation in a cell type specific manner

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Abstract
Purpose Most anticancer drugs show their antiproliferative and cytotoxic activity via induction of apoptosis. In the present study we assessed the implication and role of cordycepin, a polyadenylation-specific inhibitor and a well-known chemotherapeutic drug, in apoptosis, induced by the anticancer drug etoposide.
Methods For this purpose, a variety of leukemia and lymphoma cell lines (U937, K562, HL-60, Daudi, Molt-4) were treated with the anticancer drugs etoposide and/or cordycepin and assessed for poly(A) polymerase (PAP) activity and isoforms by the highly sensitive PAP activity assay and western blotting, respectively. Induction of apoptosis was determined by endonucleosomal DNA cleavage, DAPI staining, caspase-6 activity assay and $\Delta\Psi_m$ reduction, whereas cytotoxicity and cell cycle status were assessed by Trypan blue staining, MTT assay and flow cytometry.
Results and conclusions The results showed that PAP changes in all cell lines, in response to apoptosis induced by etoposide, in many cases even prior to hallmarks of apoptosis (endonucleosomal cleavage of DNA, $\Delta\Psi_m$ reduction). A further elucidation to this apoptosis–polyadenylation correlation was added, by cell treatment with cordycepin, resulting in either suppression (U937, K562) or induction (HL-60) of the apoptotic process, according to the cell type. However, inhibition of polyadenylation did not influence the cell lines Daudi and Molt-4 used, where alternative apoptotic pathways are induced through cleavage of DNA into high molecular weight fragments.

Keywords Cancer · Cordycepin · Etoposide · Leukemia · PAP · Anticancer drugs

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Introduction

The realisation that cancer is the result of an imbalance among cell cycle progression and cell death [1–3], led cancer research to be increasingly focused on cell cycle and cell death regulatory mechanisms. Posttranscriptional modifications seem to be increasingly responsible for the regulation–dysregulation of many cellular processes, including apoptosis. Apoptosis is a genetically controlled form of cell death, with a distinctive role in both the development and prevention of cancer [4, 5], being induced by a variety of anticancer drugs [6–10].

Recently, a first relation among polyadenylation and apoptosis has been observed. Polyadenylation occurs as a two-step reaction, the endonucleolytic cleavage of the pre-mRNA at the poly(A) site and the addition of a poly(A) tail to the 3′end of the upstream cleavage product, determining the stability and the translational ability of mRNAs [11, 12]. Among the basic proteins of the polyadenylation complex, the most studied one is polyadenylate polymerase (PAP), an RNA polymerase existing both in the nucleus and in the

cytoplasm [12, 13] with multiple isoforms (PAP I, PAP II: 80, 100, 106, 110 kDa, PAP III, PAP IV, TPAP, neo-PAP, PAPγ) [14–18]. Three different mechanisms are responsible for their generation, which include: gene duplication, alternative mRNA processing and posttranslational modifications [17]. It is possible that the human PAP gene family may expand even more in the near future and it is possible that different PAP isoforms may have applicability as biomarkers in cancer and other chronic and acute diseases. [19]. The heterogeneity of PAP advocates for a wider biological role of the enzyme in the cell and implies specialized roles for the various isoforms in different cell functions, such as cell proliferation and cell death, ensuring more precise and strict, quantitative control of PAP levels in different tissues and/or cell growth states, as well as applicability of PAP forms as biomarkers in cancer and other chronic and acute diseases [19].

The PAP, being an important unit of the polyadenylation process, is itself a target of very strict regulation. Mammalian PAP is hyper-phosphorylated during the M phase by the p34cdc2-cyclin B complex at multiple consensus and nonconsensus sites in its carboxyl-terminus serine- and threonine-rich region, that leads to inhibition of the catalytic activity of PAP [20, 21]. In addition, hypophosphorylation of PAP and increased polyadenylation activity are associated with human immunodeficiency virus type 1 Vpr expression [22].

The PAP is tightly regulated within the cell cycle via phosphorylation/dephosphorylation [27–30], with its modulations reflecting the proliferative status of the cell [23–25]. It is characteristic that highly and sustaining metabolizing lymphocytes [24], as well as less differentiated cells, have higher levels of PAP activity [25, 26]. In addition, overexpression of PAP mRNA has been demonstrated in chronic leukemias [27–29], as well as in a number of different human tumors originating in breast and pancreas, compared to the expression levels in their normal tissue counterparts [30–32]. Such an implication of PAP differential modulations in cancer was further sustained with the observation that high PAP activity levels, detected in the cytosol of breast tumors from 62 untreated patients, were associated with the TNM stage of the disease, node invasiveness and *c-erbB-2* overexpression [30]. These and additional experiments on breast cancer patients [31] suggested that PAP may be considered as a new independent unfavorable prognostic factor for early recurrence and death in breast cancer patients. These findings, coupled with the identification and overexpression of neo-PAP, a new human nuclear PAP isoform in human neoplasms, encoded by a previously uncharacterized gene [17], suggest that PAP may represent an aberrantly regulated factor in cancer cells that supports the main characteristics of their malignant phenotype, such as enhanced proliferative activity and diminished cell turnover via apoptosis. Such a hypothesis was quite intriguing for further research, especially because of the realization that cancer is the result of cells dysregulated ability to die and that most anticancer agents, alone or in combination, express their anticancer effect via apoptosis induction.

The PAP modulations along the apoptotic process refer to dephosphorylation, proteolysis and activity downregulation early in the apoptotic process (U937 cells), induced with physical agents such as heat shock or nutrient deprivation [33]. Similar modifications of PAP were also observed in preliminary results after apoptosis induction in WISH, HeLa and U937 cells, with two chemotherapeutic drugs, 5′-fluorouracil and tamoxifen [34–36]. In addition, PAP modulations in Daudi and Molt-3 cells treated with etoposide were found to be mutually associated with morphological evidence of apoptosis [37], whereas PAP modulations in human epithelioid cervix and breast cancer cell lines, treated with etoposide or cordycepin, followed cell cycle rather than apoptosis induction [38]. There are also data concerning interaction of PAP with proteins that play a major role in the apoptotic process, and influence both PAP activity and isoforms (the virus HIV protein Vpr [22], the U1 70 kDa protein of spliceosome [39] and poly(A)-binding protein (PABP) [40]).

It is also well known that most mammalian genes involved in cell cycle and apoptosis are tightly regulated at the transcriptional and posttranscriptional level. Recently, it was found that mutations of the characteristic yeast, cytoplasmic poly(A) polymerase, Cid13, result in sensitization of cells to drug-induced apoptosis, whereas its overexpression results in development of cell resistance to apoptosis [41]. Furthermore, in male mice, the absence of a testis-specific, cytoplasmic poly(A) polymerase gene (TPAP) results in incomplete elongation of poly(A) tails of particular transcriptional factor mRNAs, as well as in arrest of spermatogenesis, increased apoptosis in germ cells and infertility [42]. In addition, transgenic expression of the TPAP gene in TPAP-deficient mice resulted in resumption of normal spermiogenesis [43]. The last few years, for an increasing number of genes which are involved in the course of apoptosis, revealed that their expression is regulated via stabilization/destabilization of mRNA, such as *bcl2* and *p53* [44, 45].

The data mentioned, strongly support the idea that post-transcriptional modulations are dynamically involved in the course of cancer cell death (apoptosis) induced by anticancer agents. Therefore, the implication of polyadenylation factors in apoptosis, the cross-correlation among PAP enzyme modulations and apoptosis, and the clinically evaluated significance of cellular PAP levels in cancer prognosis and therapy, in conjunction with the fact that most anticancer agents express their anticancer action via

apoptosis induction, elevate a large number of questions and make even more important the need for further research of PAP implication in the apoptotic process, cancer prognosis and therapy.

In the present study, we initially detected early modulations of PAP activity and isoforms in a variety of different leukemia cell types (HL-60, U937, K562, Daudi, Molt-4), as a response to anticancer drug-induced apoptosis and attempted to further elucidate the revealing correlation among PAP modulations and apoptosis by using the polyadenylation-specific inhibitor 3′-deoxyadenosine (cordycepin). We, overall, aim at the recognition of PAP as a potential marker of apoptosis in cancer therapy.

Materials and methods

Cell cultures

HL-60 (human acute myeloid leukemia), U937 (human histiocytic lymphoma), K562 (human chronic myeloid leukemia in blast crisis), Molt-4 (human T-cell acute lymphoblastic leukemia) and Daudi (human Burkitt lymphoma) cell lines were maintained in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 200 U/ml penicillin, 100 μg/ml streptomycin, 0.3 g/ml L-glutamine and 2 mM $NaHCO_3$ in an atmosphere of 95% air/5% CO_2 with 100% humidity at 37°C. Cells were seeded at 4×10^5 cells/ml, incubated at 37°C and 48 h later were treated with etoposide and/or cordycepin, while in exponential growth phase, for the indicated time periods. Etoposide (Sigma Chemical Co, St. Louis, MO, USA) was prepared as a 102 mM stock in DMSO and stored at -20°C. During experiments, control cultures received equivalent solvent treatment (DMSO; 0,03%). Cordycepin (Sigma Chemical Co) was prepared as a 1 mg/ml stock in distilled H_2O and stored at -40°C. Each drug was added to the cell medium, where it remained constantly for the indicated time periods.

Preparations of cell extracts

After treatment, cells (10^7 cells) were washed twice in PBS, resuspended in lysis buffer (200 μl; 1% Nonident P-40, 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.5 mg/ml aprotinin; Sigma Chemical Co), incubated for 45 min at 4°C, centrifuged at 15,000*g* for 5 min, and stored at −70°C until further use [39].

DNA fragmentation assay

Samples (10^6 intact cells/sample) were subjected to electrophoresis on 2% agarose (Sigma Chemical Co) gel, according to Eastman protocol [46]. High-molecular weight (HMW) DNA fragments were trapped in or near the well, whereas the DNA fragments of low-molecular weight run and separated through the gel. The gel was stained with ethidium bromide and photographed by a Nikon F-801 SLR camera with accompanying UV filter and AGFA APX, ISO 25, B/W professional film.

DAPI staining

Etoposide- and/or cordycepin (Sigma Chemical Co) treated cells for the indicated time periods were collected, washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 50% methanol in PBS for 30 min and stored for up to 3 days at 4°C. Immediately prior to observation, fixed cells were washed twice with PBS, stained with 4′,6′-diamidine-2′-phenylindo dihydrochloride (DAPI, Sigma Chemical Co; 0.2 mM in PBS) and examined under a fluorescence microscope with excitation filter and LP 430 nm barrier filter [47].

MTT

Cells were assessed for sensitivity to etoposide to determine the concentration that was toxic to at least 50% of the cells after the indicated time periods of drug treatment. Cells (10^6), in triplicate, were treated with etoposide for the indicated time periods prior to addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co), in the presence of which they were further incubated for 4 h at 37°C. The absorbance of cell lysate solution was measured at 550 nm, and the results were expressed as the percentage (%) of treated cells versus untreated cells [48].

Immunoblotting and image analysis

About 70 μg of protein per condition were electrophoresed on a 7.5% polyacrylamide gel, blotted on to a nitrocellulose membrane and probed with polyclonal PAP antiserum (1:2,000 dilution), raised against cDNA made bovine PAP expressed in *Escherichia coli* and purified. PAP antiserum was a generous gift from Dr. W. Keller's laboratory. Detection was made using 1:1,000 of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co) and the immunocomplexes were visualized using 5′-bromo-4′-chloro-3′-indolyl-phosphatase (BCIP; Sigma Chemical Co) and nitro blue tetrazolium (NBT; Sigma Chemical Co) as the alkaline phosphatase substrate. The molecular weight markers used were as follows: β-galactosidase *E. coli* (116 kDa), phosphorylase b-rabbit muscle (97.4 kDa) and albumin bovine plasma (66 kDa) (Sigma Chemical Co) [49, 50]. Protein concentration was determined by the

Lowry method [51]. Polyacrylamide gel band analysis was performed using the NIH Image program, developed at the U.S. National Institute of Health (http://www.rsb.info.nih.gov/nih-image/).

PAP enzyme assay

The cell extract was added to the standard PAP assay mixture, as previously described [52]. The optimal conditions were obtained for saturating ATP levels (Sigma Chemical Co) and protein concentrations of 0.2 mg/ml to be assayed. The procedure was followed as previously described. After a kinetic analysis of the method, it was observed that in cell extracts with protein concentration of 1 mg/ml, the detection limit of PAP enzyme assay is 5 U. One unit of PAP activity is defined as the quantity of enzymes able to incorporate 1 nmol of [^{3}H]AMP (Amersham Corp.) per hour at 37°C [52].

Flow cytometry

Cell cultures, 48 h old, were incubated with etoposide and/or cordycepin, and the cell cycle was analyzed immediately after the end of drug treatment, by Becton Dickinson FACs Scan, according to propidium iodide (PI; Sigma Chemical Co) protocol [53, 54].

Mitochondrial transmembrane potential ($\Delta\Psi_m$)

Cell cultures, 48 h old, were incubated with etoposide and/or cordycepin for the indicated time periods. Cells (10^6 cells/ml) were then further incubated with 10 µg/ml JC1 (Bioproducts) in the dark for 10 min, washed twice with PBS (1×) and resuspended in 400 µl PBS (1×). The samples (10,000 cells/sample) were analyzed by Becton Dickinson FACs Scan with excitation by a 488-nm-argon-ion laser and detection of the JC1-monomer and JC1-aggregates separately in the conventional flow cytometer FL1 (green fluorescence) and FL2 (red fluorescence) channels, respectively [55, 56].

Caspase-6 activity of cell extracts

Cells (10^6/sample) were washed twice with PBS (1×) and resuspended in 200 µl of extraction buffer (10 mM HEPES/KOH buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF). After repeated freezing and thawing, cell debris were removed by centrifugation at 13,000*g* at 4°C for 20 min. 50 µl of the supernatants were incubated at 37°C for 1 h with HEPES-buffer (20 mM HEPES, pH 7.5, 10% glycerol, 4 mM DTT), containing 0.2 mM of Ac-AEVD-AFC (Ac-Ala-Glu-Val-Asp-AFC; caspase-6 fluorogenic substrate; Alexis Biochemicals), and the cleavage of the peptide was analyzed on spectrophotometer with excitation at 380 nm and emission at 505 nm. Caspase activity was expressed as percentage (%) of the apoptotic cells versus the untreated cells. Substrate stock solution: 20 mM in DMSO, stored at 20°C [57].

Results

Effect of etoposide on different leukemia cell types: cell growth, cell cycle, apoptosis

The effect of etoposide on different human leukemia cells (U937, HL60, K562, Daudi and Molt-4), exposed to 20 µg/ml of the drug for the indicated time periods, was examined and cell viability was determined by MTT assay. Figure 1a shows that the growth of all cell types was negatively affected by etoposide in a dose- (data not shown) and time-dependent manner. However, the sensitivity of cells to the cytotoxic effect of etoposide was different among the cells tested, indicating cell type dependence. Therefore, the following order of sensitivity to etoposide anti-proliferative effect is clear: HL-60 > Molt-4 > U937 > Daudi > K562.

As MTT method assesses quantitatively cell viability but cannot distinguish between growth arrest, apoptosis or necrosis, we also analyzed cell cycle arrest, DNA fragmentation, caspase-6 activity, reduction in mitochondrial transmembrane potential ($\Delta\Psi_m$), as well as the percentage (%) of trypan blue +ve cells (necrosis) (data not shown) in all the cell lines mentioned earlier.

Cell cycle arrest To further investigate the anti-proliferative mechanism of etoposide, the cell cycle phase distribution of all cell types, treated with 20 µg/ml of etoposide, was analyzed by flow cytometry. After the indicated treatments, G1 cell cycle arrest was detected in HL-60, U937 and K562 cells (Fig. 1b), whereas no alteration of any cell cycle phase was observed in Daudi and Molt-4 cells (Fig. 1b).

DNA fragmentation Typical morphological changes of apoptosis, such as chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies, were also observed in all the cell lines examined after the indicated time periods for each cell line (Fig. 1b, c), and the percentage of apoptotic cells, assessed by DAPI staining, was found to increase in a time-dependent manner. Apoptosis was further confirmed by the characteristic pattern of nucleosome-sized ladders of DNA fragments (indicative of apoptosis). DNA laddering was detected, after the indicated treatment with etoposide, in HL60, U937 and K562 cells. Interestingly, etoposide was found to induce only HMW DNA fragmentation

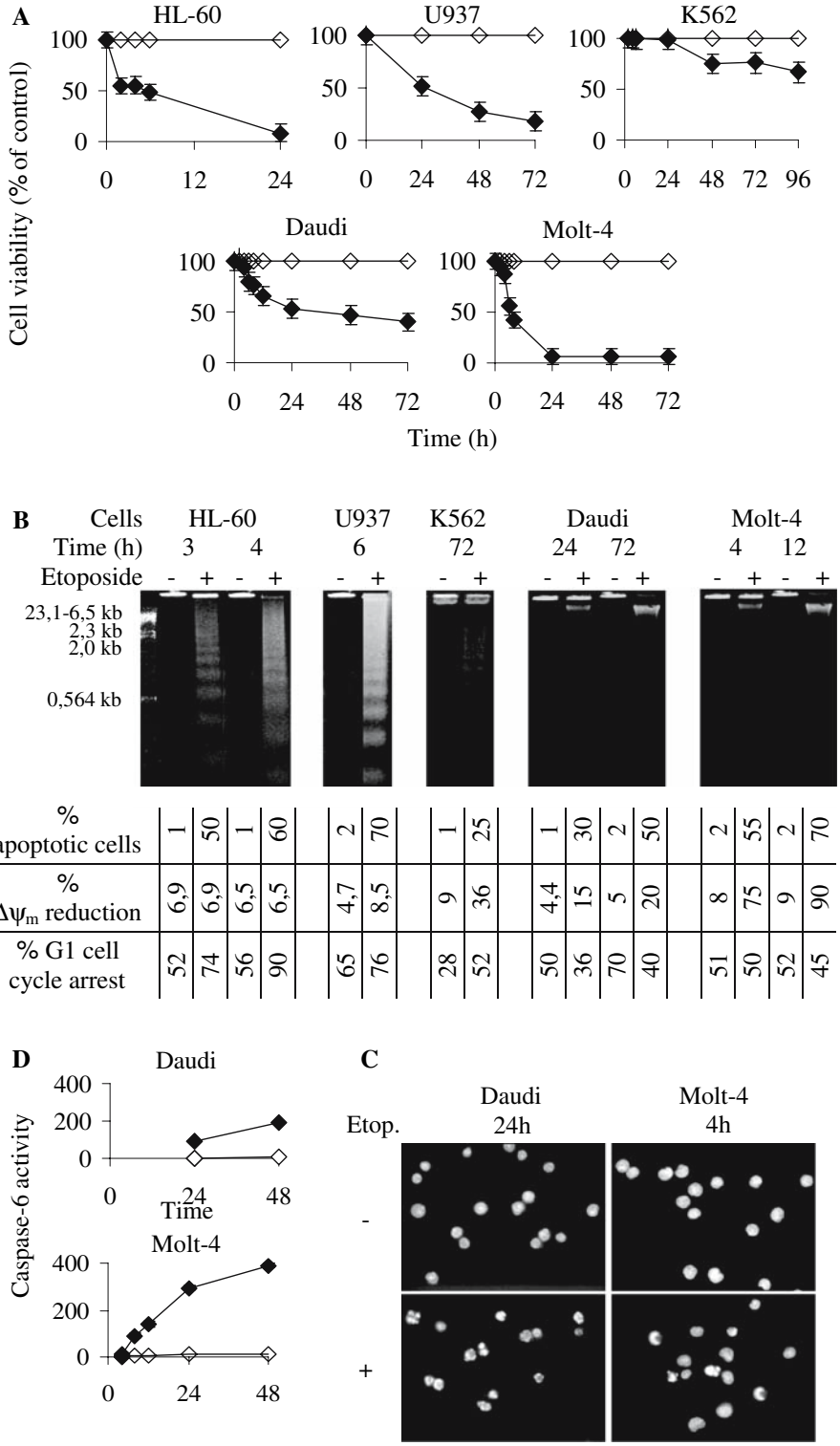

Fig. 1 **a** Cell viability, as assessed by the MTT assay, **b** DNA cleavage, % of apoptotic cells as counted by DAPI staining, % of $\Delta\psi_m$ reduction, % of G1 cell cycle arrest, **c** DAPI staining (magnification, ×400) and **d** caspase-6 activity, of HL-60, U937, K562, Daudi and Molt-4 cells, in the absence (*open diamond*, −) or presence (*filled diamond*, +) of 20 μg/ml of etoposide (Etop), for the indicated time periods. Data points are the mean of three separate experiments

(as assessed by both agarose gel electrophoresis and DAPI staining) in Daudi and Molt-4 cells (Fig. 1b, c). Induction of apoptosis in both cell types was further confirmed with assessment of caspase-6 activity (effector caspase, being activated in all apoptotic pathways) (Fig. 1d), which was found at high levels in both Daudi and Molt-4 cells.

$\Delta\Psi_m$ To further distinguish apoptotic pathways induced by etoposide in each cell line examined, we also assessed changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$), an early marker of mitochondrial apoptotic pathway. The results showed that $\Delta\Psi_m$ was almost unaffected in HL-60, U937 and Daudi cells, whereas in K562 and Molt-4 cells it was highly reduced (Fig. 1b).

Taken together, the earlier results demonstrate that all five cell lines examined are induced to apoptosis with 20 μg/ml etoposide and therefore the observed variation in cell viability reflected mainly apoptosis. However, apoptosis was induced probably via distinct pathways in different cell types and with distinct sensitivity to the apoptotic, anti-proliferative and cytototoxic effect of the agent. Therefore, the response of different cell types distinguish them into two major groups:

1. Cells induced to apoptosis via the characteristic endonucleosomal cleavage of DNA at different time periods of exposure to etoposide: HL-60, U937, K562.
2. Cells induced to apoptosis via nuclear fragmentation and DNA cleavage into HMW fragments, without the characteristic endonucleosomal DNA cleavage: Molt-4, Daudi.

PAP modulations in distinct leukemia cell types without any treatment

To observe the possible discrepancies in PAP activity and isoform levels among the leukemia cell types used, total cellular extracts from each cell type, without any treatment, were assayed for PAP activity and isoforms. The results (Fig. 2a) showed that PAP activity and isoform levels are quite different among the cell types, with Daudi possessing the lowest levels of the enzyme and Molt-4 the highest.

PAP modulations along with etoposide-induced apoptosis, in distinct leukemia cell types

To evaluate the involvement of PAP, an enzyme only recently related to apoptosis [33–36, 41], in the mechanism of etoposide-induced apoptosis in cancer cells, we examined the modulations in protein expression and enzyme activity of PAP. Treatment of all cell lines with 20 μg/ml of etoposide for the indicated time periods resulted in changes of both PAP forms and activity along the process of apoptosis and in some cases even prior to early apoptotic hallmarks (Fig. 2b; Table 1), such as endonucleosomal cleavage of DNA, morphological changes of nucleus and modulations in $\Delta\Psi_m$ in accordance with the cell type. The phosphorylated forms of PAP (100 and 106 kDa) were the first that responded to etoposide treatment, in all the cell types examined (Fig. 2b; Table 1). PAP modulations also showed great discrepancies among the different cell lines, but were firm and representative of each cell type. Taken together, our results demonstrate a correlation among PAP modulations and apoptosis, which is cell-type specific.

The polyadenylation inhibitor cordycepin suppresses etoposide-induced apoptosis

In an attempt to elucidate the correlation observed among PAP modulations and apoptosis, we exposed different cell types (U937, K562, Daudi, Molt-4) to a polyadenylation-specific inhibitor, 3′-deoxyadenosine (cordycepin), and apoptosis induction was assessed, as well as modulations of PAP activity and forms. The results were as follows:

U937 In U937 cells, treatment was performed with 20 μg/ml of etoposide-induced apoptosis after 6 h of exposure. When cells were preincubated with 20 μg/ml of cordycepin for 3–24 h, and then treated with 20 μg/ml of etoposide (in the presence of cordycepin), a gradual inhibition of both endonucleosomal cleavage of DNA (Fig. 3a) and cytotoxic

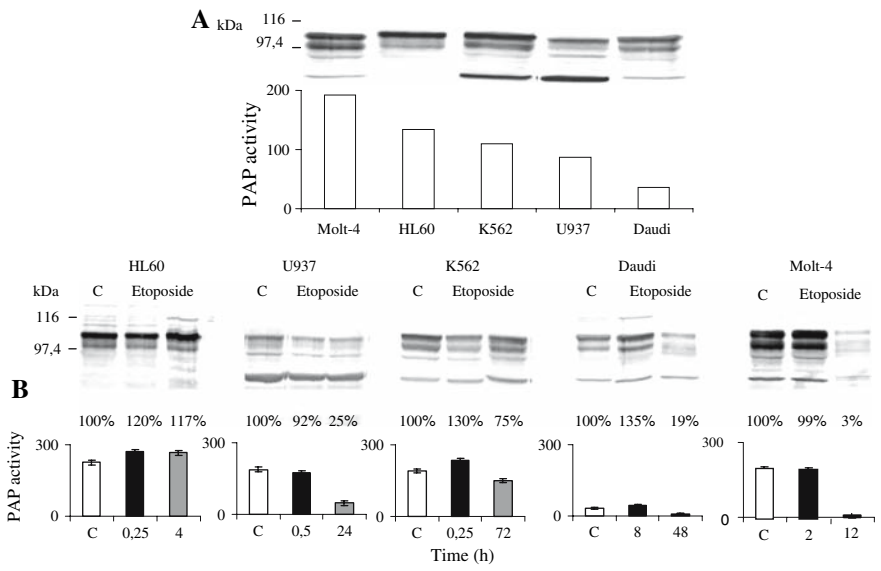

Fig. 2 Activity and western blot analysis of PAP enzyme forms in lysates from **a** control samples (without any treatment), and **b** samples after treatment of HL-60, U937, K562, Daudi and Molt-4 cells in the absence, **c** or presence of 20 μg/ml etoposide, for the indicated time periods. The *numbers* (%) represent the percentage of control PAP activity after treatment with etoposide for the indicated time periods. *Black shaded bars* represent time periods before appearance of DNA cleavage, whereas *gray shaded bars* represent time periods along DNA fragmentation

Table 1 PAP form modulations along with etoposide-induced apoptosis, in distinct leukemia cell types

PAP form molecular weight (kDa)	Time of exposure (h) to 20 μg/ml etoposide					
	HL-60					
	0		0.25		4	
	F	SD	F	SD	F	SD
106	1	0.001	−1.12	0.055	1.33	0.09
100	1	0.02	−1.28	0.07	−1.49	0.08
	U937					
	0		0.5		24	
	F	SD	F	SD	F	SD
106	1	0.003	**−1.75**	0.02	**−2**	0.07
100	1	0.01	**−2.56**	0.03	**−3.85**	0.4
80	1	0.001	−1.09	0.008	−1.07	0.06
	K562					
	0		0.25		72	
	F	SD	F	SD	F	SD
106	1	0.001	**−1.56**	0.04	1.11	0.1
100	1	0.004	**−1.88**	0.04	1.02	0.08
	Daudi					
	0		8		48	
	F	SD	F	SD	F	SD
106	1	0.009	1.44	0.04	**−3.85**	0.9
100	1	0.01	**1.67**	0.05	**−3.33**	0.09
80	1	0.001	**2.17**	0.05	−1.19	0.01
	Molt-4					
	0		2		12	
	F	SD	F	SD	F	SD
106	1	0.0001	1.17	0.04	**−7.14**	0.35
100	1	0.01	−1.09	0.003	**−16.66**	0.8
80	1	0.0008	−1.07	0.07	**−2.63**	0.09

Numbers in bold represent significant modulations (>1.5-fold) in PAP forms. A positive number indicates an increase in the level of PAP isoform expression; a negative number indicates a decrease in the level of PAP form expression

SD standard deviation, *F* average ratio of change (*n*-fold)

effect (Fig. 3b) of etoposide was observed, which was strictly dependent on both the preincubation time with cordycepin as well as on the concentration of the polyadenylation inhibitor used (data not shown). PAP response, to the combination of cordycepin and etoposide, revealed an antagonistic effect among etoposide and cordycepin, reflected upon PAP modulations, with the former decreasing or not affecting both PAP forms and activity, according to time of exposure, whereas the latter increases them significantly (Fig. 3c; Table 2). Combination of them led to predominance of cordycepin's effect and increase of both PAP forms and activity, as compared to untreated cells (Fig. 3c; Table 2).

K562 Although higher concentration of cordycepin is required in the case of K562 cells, when they were preincubated with 60 μg/ml cordycepin for 8 h, and then treated with 20 or 60 μg/ml etoposide (in the presence of cordycepin) for 72 h, inhibition of both endonucleosomal cleavage of DNA (Fig. 4a), $\Delta\Psi_m$ reduction (Fig. 4b) and the cytotoxic effect (as assessed by both MTT and the trypan blue exclusion method) of etoposide (Fig. 4c) was also observed, which was strictly dependent on the preincubation time with cordycepin as well as on the concentration of the polyadenylation inhibitor used (data not shown). PAP response, to the combination of cordycepin and etoposide, differs between U937 and K562 cells, probably because of the involvement of different apoptotic pathways. In the case of K562 cells, PAP modulations involve an increase in the levels of the phosphorylated forms (100 and 106 kDa) and density after exposure to either etoposide or cordycepin alone, or the combination of them (Fig. 4d; Table 3), whereas PAP activity decreases in all cases (Fig. 4d).

Daudi and Molt-4 Treatment of Molt-4 and Daudi cells with combination of cordycepin and etoposide did not affect either the cleavage of DNA into HMW fragments or PAP modulations, induced by etoposide alone (data not shown).

HL-60 and cordycepin: an exceptional model of apoptosis induction

When HL-60 cells were treated with 20 to 60 μg/ml cordycepin for the indicated time periods, we confronted an unexpected result. Exposure of HL-60 cells to the polyadenylation inhibitor cordycepin led to apoptosis through induction of endonucleosomal cleavage of DNA since 8 h of exposure (Fig. 5a: *Left*), as well as $\Delta\Psi_m$ reduction since 4 h (Fig. 5b). Pretreatment of HL-60 cells for 2 h with specific caspase inhibitors involved in distinct apoptotic pathways (according to Alexis Biochemicals protocol) revealed that the apoptotic pathway activated after exposure of HL-60 cells to cordycepin is caspase-2 (Z-VD(Ome)-VAD(Ome)-FMK; Z-Val-Asp(Ome)-Val-Val-Asp(Ome)-FMK), -8 (Z-LE(Ome)-TD(Ome)-FMK; Z-Leu-Glu(Ome)-Thr-Asp(Ome)-FMK) and -10 (Z-AEVD-FMK; Z-Ala-Glu-Val-Asp-FMK) dependent (Fig. 5c), because they partly inhibited endonucleosomal cleavage of DNA induced by cordycepin alone. Those caspases were also found to be involved in the

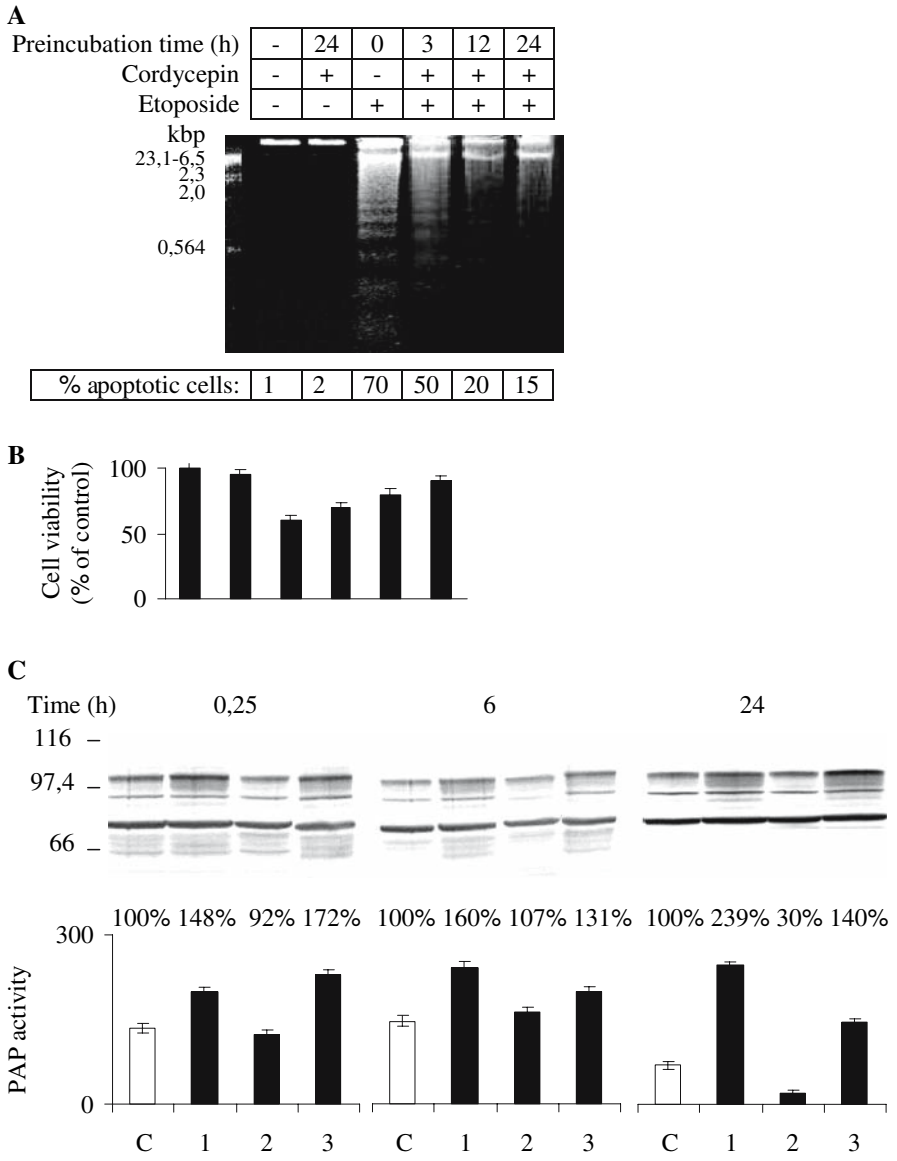

Fig. 3 **a** DNA cleavage and % of apoptotic cells as counted by DAPI staining, **b** Cell viability, as assessed by the MTT assay, after preincubation of U937 cells for different time periods with 20 μg/ml cordycepin, followed by incubation with 20 μg/ml etoposide (in the presence of cordycepin) for 6 h. **c** Activity and western blot analysis of PAP enzyme forms in lysates from U937 cells, after: *C* No teatment, *1* incubation with cordycepin, *2* incubation with etoposide, *3* preincubation of cells with 20 μg/ml cordycepin for 24 h, followed by incubation with 20 μg/ml etoposide (in the presence of cordycepin) for 0,25, 6 and 24 h. *Numbers* represent the percentage (%) of control PAP activity after the indicated cell treatment. Data points are the mean of three separate experiments

apoptotic pathway, induced by cordycepin, prior to mitochondria, as pretreatment of HL-60 cells with their inhibitors led to partial inhibition of $\Delta\Psi_m$ reduction induced by cordycepin alone (Fig. 5d). As far as PAP modulations is concerned, a continuous decrease of enzyme activity is observed in as early as 15 min of treatment with cordycepin, whereas PAP forms show significant decrease mainly since 1 h of exposure (Fig. 5e; Table 4), with the 106 kDa decreasing first and the 100 kDa decreasing along DNA cleavage (8 h of exposure to etoposide).

Discussion

The response of different leukemia cell types to etoposide, separated them into two groups: (1) Specific leukemia and lymphoma cell lines (HL-60, U937, K562) induced to apoptosis via endonucleosomal cleavage of DNA at different time periods of exposure to etoposide. (2) Lymphoma cells (Molt-4, Daudi) which are induced to apoptosis via nuclear fragmentation and DNA cleavage into HMW fragments, without the characteristic endonucleosomal DNA cleavage.

The induction of apoptosis with delay in K562 cells or via alternative apoptotic pathways in Daudi and Molt-4 cells, where classic apoptotic indicators (endonucleosomal degradation of DNA, caspase activation) are either not detected or emerged with delay, could lead to a misleading interpretation of the apoptotic capability of various anticancer drugs or the characterization of cancer cells as resistant to the apoptotic process. Therefore, the identification of new molecules, involved in apoptosis, detected quite early and independently of endonucleosomal DNA fragmentation,

Table 2 PAP form modulations along suppression of etoposide-induced apoptosis with the polyadenylation inhibitor cordycepin, in U937 cells

PAP form molecular weight (kDa)	Time of exposure: 0.25 h							
	C		1		2		3	
	F	SD	F	SD	F	SD	F	SD
106	1	0.005	**1.54**	0.0001	**−1.54**	0.0001	1.24	0.045
100	1	0.06	**5.73**	0.007	**−4.16**	0.0035	**3.96**	0.0003
80	1	0.003	1.05	0.1	−1.22	0.004	−1.12	0.06
	Time of exposure: 6 h							
	C		1		2		3	
	F	SD	F	SD	F	SD	F	SD
106	1	0.0037	**1.8**	0.05	1	0.055	**1.78**	0.07
100	1	0.004	**19.94**	0.7	1.5	0.045	**9.49**	0.8
80	1	0.0001	1.16	0.06	−1.06	0.2	1.01	0.4
	Time of exposure: 24 h							
	C		1		2		3	
	F	SD	F	SD	F	SD	F	SD
106	1	0.0003	**1.76**	0.07	−1.03	0.06	**2.08**	0.09
100	1	0.005	**7**	0.34	1.37	0.04	**10.05**	0.5
80	1	0.0009	1.1	0.05	1.11	0.08	1.13	0.05

Numbers in bold represent the significant modulations (>1.5-fold) in PAP forms. A positive number indicates an increase in the level of PAP form expression; a negative number indicates a decrease in the level of PAP form expression

PAP enzyme forms of U937 cells, after: (C) No treatment, (1) incubation with cordycepin, (2) incubation with etoposide, (3) preincubation of cells with 20 μg/ml cordycepin for 24 h, followed by incubation with 20 μg/ml etoposide (in the presence of cordycepin) for 0.25, 6 and 24 h

SD standard deviation, *F* average ratio of change (*n*-fold)

as well as of caspase activation, seems to have further interest for the elucidation of drug effectiveness (apoptosis induction) and cancer therapy.

With regard to PAP response to etoposide, it emerges that the enzyme is under a very characteristic regulation which is mainly cell-type specific. PAP modulations seem to be under a delicate and strict control mechanism, occurring, in three out of five cell lines (U937: 0.5 h, K562: 0.25 h, Daudi: 8 h), very early in the apoptotic process, with the phosphorylated isoforms of PAP being the first that respond to etoposide treatment, in all the cell lines examined. However, the differences observed among the cell lines are firm and representative of each cell type, revealing the possibility that the differential cellular content in mRNAs leads to different needs for polyadenylation in each cell type, in order for the apoptotic signal to be induced and transmitted. This may be responsible for the diverge cellular levels of PAP forms and activity in the different cell types used, confirming a mutual, although cell type-dependent, correlation among PAP modulations and apoptosis. This is also supported by the fact that PAP isoforms and activity levels are quite different even at control cellular extracts, where no apoptosis induction has occurred, ensuring that the polyadenylation requirements of each cell type are different. It is also possible, the induction of differential apoptotic pathways to be in part responsible for the diverged PAP response to etoposide treatment, among the cell lines used.

In an attempt to further elucidate this correlation, we inhibited polyadenylation after cell treatment with the polyadenylation specific inhibitor, 3′-deoxyadenosine (cordycepin), which is used in the treatment of different types of leukemia [58–62], as well as many modified nucleoside analogs which have been used extensively as chemotherapeutic agents [63]. Nucleoside antimetabolites typically are taken up by cells, metabolized, and subsequently enter cellular nucleotide pools, where they exert their chemotherapeutic action. In particular, modified purine analogs have demonstrated promise for cancer treatment [63]. Short exposure (2 h) to cordycepin leads to reduction of mRNA levels in the cytoplasm, likely because of reduction of PAP mRNA levels, whereas after extensive exposure, reduction of the levels of other mRNAs occurs [59]. Therefore, it was expected that inhibition of polyadenylation would result in intervention in PAP levels and consequently changes in the apoptotic process.

The increase of PAP phosphorylated forms and activity levels since 15 min of exposure of U937 cells to cordycepin, may be due to the cell's need for polyadenylation, either because of the inhibition of polyadenylation caused by cordycepin or because of PAP isoform and activity modulations. Preincubation of cells with cordycepin followed by exposure to etoposide leads to apoptosis inhibition as well as the maintenance of PAP activity and forms at increased levels. A lot of factors could be involved in this phenomenon, such as inhibition of polyadenylation and therefore of the expression of caspases, nucleases and more generally of molecules that participate in the apoptotic pathway induced.

Treatment of K562 cells with cordycepin also leads to increase of phosphorylated forms of PAP, but to a reduction of PAP activity after 8 h of exposure. The 8 h of exposure to cordycepin inhibits both nuclear and cytoplasmic polyadenylation [64]. Therefore, preincubation of cells with cordycepin followed by exposure to etoposide leads to complete inhibition of endonoucleosomal cleavage of DNA, in the case of both U937 and K562 cells, with requirement for higher concentrations of cordycepin in the case of K562 cells. Because of the higher levels of

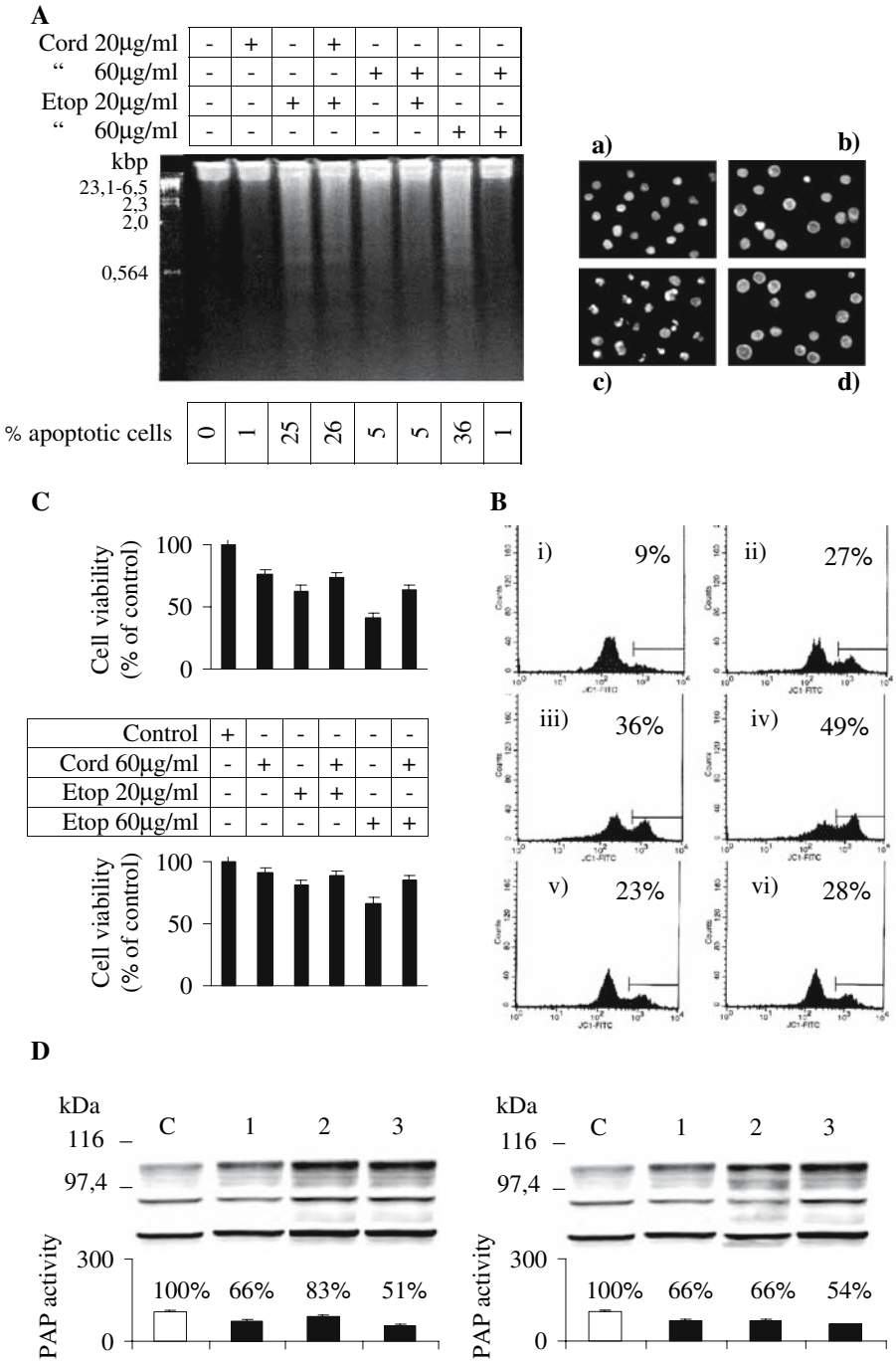

Fig. 4 **a** *Left* DNA cleavage and % of apoptotic cells as counted by DAPI staining, after preincubation of K562 cells with 20 or 60 µg/ml of cordycepin for 8 h, followed by treatment with 20 or 60 µg/ml etoposide (in the presence of cordycepin) for 72 h. *Right* DAPI staining (magnification, ×400), after: *a* no treatment, or treatment with *b* cordycepin 60 µg/ml for 80 h, *c* etoposide 20 µg/ml for 72 h, *d* cordycepin 60 µg/ml for 8 h, followed by 72 h of exposure to both cordycepin and etoposide 20 µg/ml. **b** % of mitochondrial transmembrane potential ($\Delta\psi_m$) modulations, as determined by changes in fluorescence upon JC-1 loading, after: *i* no treatment, or treatment with *ii* cordycepin 60 µg/ml for 80 h, *iii* etoposide 20 µg/ml for 72 h, *iv* etoposide 60 µg/ml for 72 h, *v* cordycepin 60 µg/ml for 8 h, followed by 72 h of exposure to either both cordycepin and etoposide 20 µg/ml, or *vi* both cordycepin and etoposide 60 µg/ml. **c** *Upper diagram* Cell viability assessed by MTT. *Lower diagram* Cell viability as assessed by trypan blue method. **d** *Left* Activity and western blot analysis of PAP enzyme forms in lysates from K562 cells, after treatment (*C*) in the absence (*white column*) or (*1*) presence (*black columns*) of 60 µg/ml cordycepin for 80 h, (*2*) 20 µg/ml etoposide for 72 h, (*3*) 60 µg/ml cordycepin for 8 h, followed by 72 h of exposure to both cordycepin and 20 µg/ml etoposide. *Right* Activity and western blot analysis of PAP enzyme forms in lysates from K562 cells, after treatment (*C*) in the absence (*white column*) or (*1*) presence (*black columns*) of 60 µg/ml cordycepin for 80 h, (*2*) 60 µg/ml etoposide for 72 h, (*3*) 60 µg/ml cordycepin for 8 h, followed by 72 h of exposure to both cordycepin and 60 µg/ml etoposide. Data points are the mean of three separate experiments

Table 3 PAP form modulations along suppression of etoposide induced apoptosis with the polyadenylation inhibitor cordycepin, in K562 cells

PAP form molecular weight (kDa)	C		1		2		3	
	F	SD	F	SD	F	SD	F	SD
106	1	0.0008	**1.67**	0.1	**1.7**	0.15	**1.62**	0.1
100	1	0.0004	**3.10**	0.5	**2.63**	0.4	**2.33**	0.09
80	1	0.0007	1.15	0.2	1.16	0.1	1.11	0.1
	C′		1′		2′		3′	
	F	SD	F	SD	F	SD	F	SD
106	1	0.0001	**1.77**	0.05	**1.95**	0.05	**2.72**	0.05
100	1	0.00001	**3.32**	0.3	**8.70**	0.6	**7.61**	0.3
80	1	0.004	1.22	0.2	1.08	0.06	**1.69**	0.07

Numbers in bold represent the significant modulations (>1.5-fold) in PAP isoforms. A positive number indicates an increase in the level of PAP form expression; a negative number indicates a decrease in the level of PAP form expression

PAP enzyme forms of K562 cells, after treatment (C) in the absence or (1) presence of 60 μg/ml cordycepin for 80 h, (2) 20 μg/ml etoposide for 72 h, (3) 60 μg/ml cordycepin for 8 h, followed by 72 h of exposure to both cordycepin and 20 μg/ml etoposide

PAP enzyme forms of K562 cells, after treatment (C′) in the absence or (1′) presence of 60 μg/ml cordycepin for 80 h, (2′) 60 μg/ml etoposide for 72 h, (3′) 60 μg/ml cordycepin for 8 h, followed by 72 h of exposure to both cordycepin and 60 μg/ml etoposide

SD standard deviation, *F* average ratio of change (*n*-fold)

adenosine deaminase that allocates in K562 cells [60], it is likely that the enzyme leads to hydrolysis and inhibition of cordycepin action before it is incorporated in mRNAs, resulting in requirement for higher concentration of cordycepin.

The inhibition of endonucleosomal cleavage of DNA by cordycepin and therefore of apoptosis in these two cell lines (U937 and K562), as well as the differential response of PAP in them, could reflect the endogenous sensitivity of these cell types to apoptosis. U937 cells constitute a cellular system with high endogenous sensitivity for apoptosis induction, and their exposure to cordycepin probably leads to repression of the polyadenylation of apoptotic genes that are influenced more intensely. In K562 cells, which constitute a model system for the study of resistance to apoptosis, treatment with cordycepin probably leads to repression of polyadenylation of survival genes, because in this case their expression is legitimate to be more intense. As far as the differential response of PAP activity towards the exposure of these two cell types to cordycepin is concerned, it is likely to be due to modulations of PAP or other factors of the polyadenylation complex, that control the expression of different genes involved in diametrically opposite courses of cell survival or death.

Moreover, incubation of HL-60 cells with cordycepin led to an unexpected observation, exceptional to the rest of the cell types, which was the induction of apoptosis. Many adenosine analogues (cordycepin, deoxyconformycin, Ara-A), individually or in combination, express their anticancer action through induction of either cell differentiation [61] or apoptosis [62], processes that are cell type dependent. In HL-60 cells, in the presence of cordycepin, the phosphorylated forms of PAP (PAPII) are decreased by the first hour of treatment. At the same time we also observe reduction of PAP activity. These changes are continued even after the endonucleosomal cleavage of DNA. This fast, intense and generalized reduction of PAP in the HL-60 cells suggests a reciprocal cross-correlation between PAP and apoptosis.

Etoposide-induced apoptosis in the HL-60 cells presents differences (rapid, intense process, PAP activity increase) from the one induced with cordycepin (slow process, continuous PAP forms and activity reduction). The observed differences and the fact that a specific polyadenylation inhibitor induces apoptosis, possibly suggest distinct apoptotic pathways induced by the two types of apoptotic inducers. The specific caspase inhibitors used revealed entanglement of caspase 2, 8 and 10, as well as changes of mitochondrial transmembrane potential ($\Delta\Psi_m$) in the apoptotic pathway induced with cordycepin, something that does not happen with etoposide. In the case of activation of the mitochondrial pathway after exposure to cordycepin, we have continuous reduction of PAP activity and forms, whereas in the case of activation of apoptotic pathways which are independent of changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$), we observe no significant effect on PAP forms. The results support the view that in the presence of cordycepin or etoposide, in the HL-60 cell line, apoptosis is induced via activation of distinct, still mitochondrial pathways.

Overall, polyadenylation, and therefore PAP, are involved in and influence anticancer drug-induced apoptosis, in cases where endonucleosomal DNA fragmentation occurs, either by inhibition (U937, K562) or induction (HL-60) of the apoptotic process, according to the cell type. However, inhibition of polyadenylation does not influence cases where alternative apoptotic pathways are induced through cleavage of DNA into HMW fragments (B-, T- cells).

In the past few years, an increasing number of genes involved in cancer development, progression and therapeutics were also found to be implicated in cancer cell apoptosis and vice versa. Such cases include PAP gene family members, as well as *BCL2* gene family members and many more [65]. Their expression profiles in many types of cancer have been examined, leading to the speculation that many of them may be of high prognostic and diagnostic value for several cancer types, requiring further research

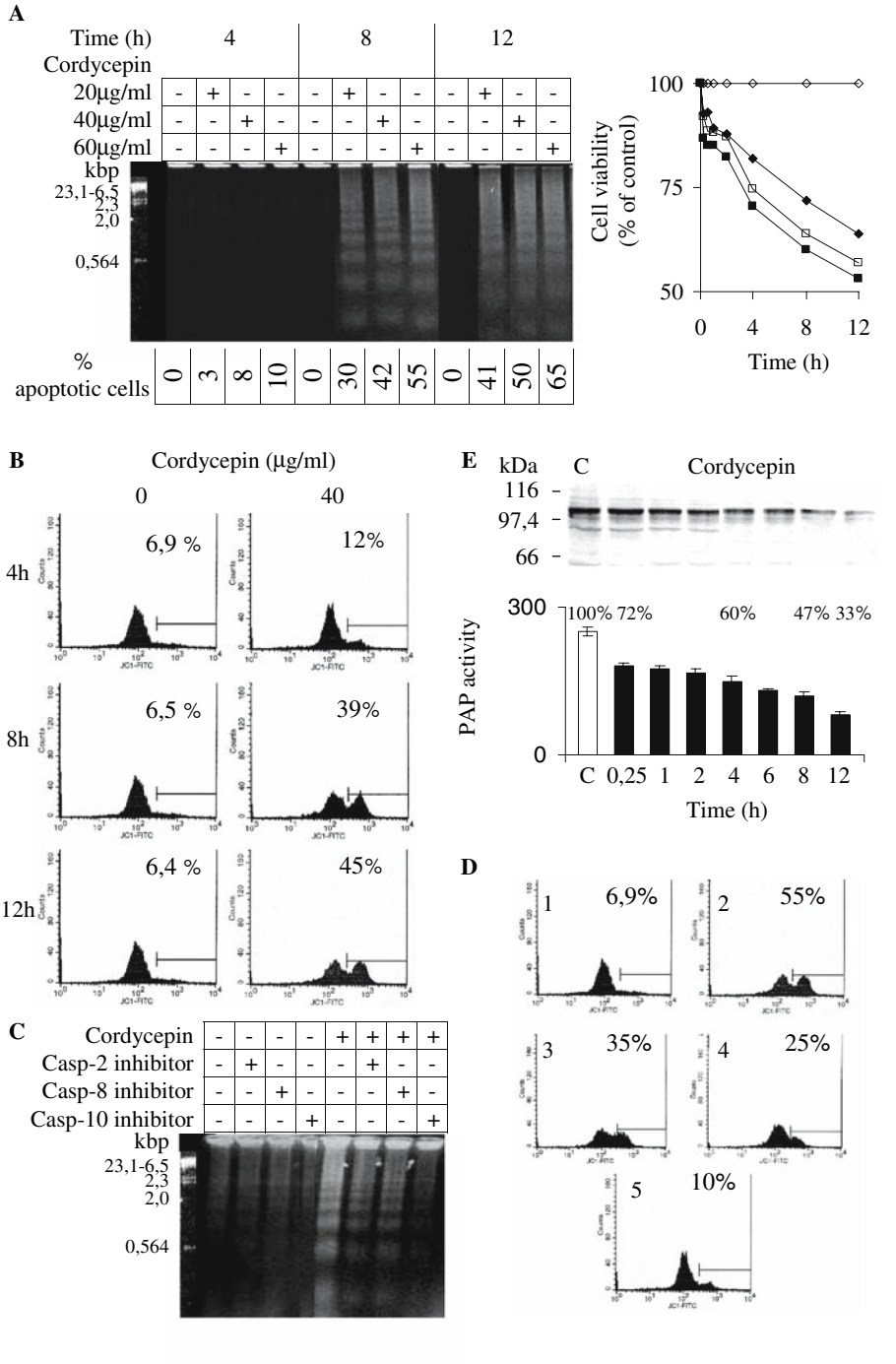

Fig. 5 Cordycepin induced apoptosis in HL-60 cells. **a** *Left* DNA cleavage, % of apoptotic cells (as counted by DAPI staining) and *Right* Cell viability of HL-60 cells, as assessed by the MTT assay, after no treatment (*open diamond* −), or treatment with 20 (*filled diamond* +), 40 (*open square* +), or 60 μg/ml (*filled square* +) of cordycepin. **b** Percentage of mitochondrial transmembrane potential ($\Delta\psi_m$) modulations, compared to control, as determined by changes in fluorescence upon JC-1 loading. **c** Inhibition of endonucleosomal cleavage of DNA by specific caspase inhibitors (Alexis Biochemicals). **d** Suppression of $\Delta\psi_m$ reduction by specific caspase inhibitors. *Numbers* represent % of mitochondrial transmembrane potential ($\Delta\psi_m$) modulations, as determined by changes in fluorescence upon JC-1 loading, in: *1* cells without any treatment, *2* cells treated with 40 μg/ml cordycepin for 18 h, *3* cells pretreated with caspase-2 inhibitor or *4* caspase-8 inhibitor or *5* caspase-10 inhibitor, for 2 h and then incubated with 40 μg/ml cordycepin for 18 h. **e** PAP enzyme forms and activity of HL-60 cells, after treatment in the absence (*white column*) or presence (*black columns*) of 40 μg/ml cordycepin. Data points are the mean of three separate experiments

and clinical evaluation [65, 66]. The *BCL2* family of proteins (including *BCL2L12*, a new member) plays an important role in moderating the cellular program of apoptosis. The levels of the various members of the *BCL2* family have been shown to have prognostic potential and to determine response to chemotherapy in breast tumours or other types of cancer, as well as in distinct cancer cell systems [65–69]. However, further research is needed to increase our understanding of the extent to which and the mechanisms by which *BCL2* family members and other families of genes, such as *PAP* gene family, are involved in cancer development, providing the basis for earlier and more accurate cancer diagnosis, prognosis, more effective evaluation of cancer transformation and more personalized therapeutic intervention that targets the apoptosis pathway, with a corresponding improvement in chemotherapy [65].

Our findings aim at the recognition of PAP as a potential marker of apoptosis in cancer therapy. The specific tissue expression and activity patterns of PAP isoforms in distinct types of cancer suggest multiple physiological roles.

Table 4 PAP form modulations along induction of apoptosis with the polyadenylation inhibitor cordycepin, in HL-60 cells

PAP form molecular weight (kDa)	HL-60															
	Time of exposure (h)															
	0		0.25		1		2		4		6		8		12	
	F	SD	F	SD	F	SD	F	SD	F	SD	F	SD	F	SD	F	SD
106	1	0.001	1.20	0.1	**−1.64**	0.03	**−1.61**	0.03	**−1.85**	0.05	**−1.85**	0.2	**−2.78**	0.4	**−5.55**	0.4
100	1	0.003	1.13	0.09	1.10	0.05	−1.05	0.06	−1.09	0.08	−1.07	0.6	**−3.85**	0.3	**−3.33**	0.9

Numbers in bold represent the significant modulations (>1.5-fold) in PAP forms. A positive number indicates an increase in the level of PAP form expression; a negative number indicates a decrease in the level of PAP form expression

SD Standard deviation, *F* average ratio of change (*n*-fold)

However, much need to be learned about the expression and activity of PAP isoforms in diverse cancer types and the biological effects resulting from their enzymatic action. It is also possible new ways of intervention to be recognized, concerning the regulation and dysregulation of polyadenylation, which may allow the observation, evaluation and exploitation of apoptosis and cancer outcome.

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