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ORIGINAL ARTICLE

The effect of the polyadenylation inhibitor cordycepin on human Molt-4 and Daudi leukaemia and lymphoma cell lines

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Abstract
Purpose Posttranscriptional modifications, such as polyadenylation, are very often implicated in the regulation and dysregulation of cell death, through regulation of the expression of specific genes. Based on the fact that an increasing number of adenosine analogues show their antiproliferative and cytotoxic activity via induction of apoptosis, we assessed the effect of cordycepin, a polyadenylation specific inhibitor, an adenosine analogue and a well-known chemotherapeutic drug, on two human leukemia and lymphoma cell lines.
Methods Cells were treated with the anticancer drug 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 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 the differentiated modulations of PAP in the two cell lines may be a result of the additive effect of the changes in cell cycle and apoptotic pathway induced.

Keywords Apoptosis · Cell viability · Cordycepin · Cytotoxicity · PAP · Polyadenylation inhibitor

Introduction

Nucleoside antimetabolites typically are taken up by cells, metabolized, and subsequently enter cellular nucleotide pools, where they exert their effect. Until recently, it was believed that the use of purine nucleosides and their analogues such as 2′chlorodeoxyadenosine (CdA), 2′deoxyadenosine (AdR), 3′deoxyadenosine (3′AdR) (cordycepin), adenosine (AR), adenine arabinoside (Ara-A), deoxyguanosine (GdR) and guanine arabinoside (Ara-G) in chemotherapeutic regimens [1] was mainly based on their antiproliferative and cytotoxic effect. However, recent studies showed that many of adenosine analogues (cordycepin, deoxyconformycin, Ara-A), individually or in combination, show their anticancer activity through induction of either differentiation [2–4] or programmed cell death [5–9].

Cordycepin (3′-deoxyadenosine) is a nucleoside derivative (analogue of adenosine) originally extracted from *Cordyceps sinensis* [10] and a well-known polyadenylation inhibitor. It has been previously reported that short exposure (2 h) to cordycepin, leads to reduction of mRNA levels in the cytoplasm, likely because of reduction of polyadenylate polymerase (PAP) mRNA levels, whereas extensive exposure to the drug leads to reduction of the levels of other mRNAs [11]. Therefore, it was expected that inhibition of polyadenylation would result in intervention in PAP levels

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and consequently changes in processes concerning cellular fate and response, such as cancer and apoptosis.

Cordycepin is known to possess distinct pharmacological activities, such as antiviral and anti- inflammatory. However, an increasing number of reports support its anticancer activity. Cordycepin is being used in the treatment of different types of leukemia [4, 6, 11–13] and it exerts antiproliferative effects on mouse melanoma and lung carcinoma cells by stimulating adenosine A3 receptors on tumor cells [14], as well as antimetastatic action on mouse melanoma cells [15]. In addition, cordycepin, in combination with pentostatin, was found to possess significant synergistic action in patients with terminal deoxynucleotidyl transferase-positive acute lymphocytic leukemia [16]. The nucleoside analogue cordycepin was also found to be substantially more cytotoxic to terminal deoxynucleotidyl transferase positive (TdT+) leukemic cells than to TdT− leukemic cells in vitro in the presence of deoxycoformycin (dCF) and has been considered as a therapeutic agent for TdT+ leukemia [5]. In addition, the apoptotic effect of cordycepin became evident in OEC-M1 human oral squamous cancer cells [8], as well as in colorectal and hepatocellular cancer cells [9]. Furthermore, the petroleum ether (PE), ethyl acetate (EtOAc) and ethanol (EtOH) extracts of the cultivated mycelium of a Cordyceps sinensis (Cs) fungus showed an important inhibitory effect on the proliferation of four cancer cell lines, MCF-7 (breast cancer), B16 (mouse melanoma), HL-60 (human premyelocytic leukaemia) and HepG2 (human hepatocellular carcinoma) [7]. The EtOAc extract, in particular, which contained carbohydrates, adenosine, ergosterol and cordycepin, had the most compelling effect against all cancer cell lines [7].

Recently, a series of data suggested a first relation among polyadenylation and apoptosis [17–22]. Polyadenylation is, among others a crucial phase in the regulation of gene expression, determining mRNA stability and efficient translation after export to the cytoplasm [23–28]. The most studied protein of the polyadenylation complex is polyadenylate polymerase (PAP), an RNA polymerase [29, 30] with multiple forms [23–27]. Such a heterogeneity advocates for its wider biological role in the cell and implies its specialized roles in different cell functions, such as cell proliferation and cell death, as well as the possible value of PAP forms as biomarkers in cancer [26, 28, 31–40].

As far as the role of PAP in leukaemia is concerned, bibliographic data have shown that highly and sustaining metabolizing lymphocytes [32], as well as less differentiated cells show higher levels of PAP activity [33, 34]. In addition, overexpression of PAP mRNA has been demonstrated in chronic leukemias [35–37]. These findings, coupled with the identified modulations of PAP in distinct human neoplasms [38, 39], suggest an aberrantly regulated role for PAP in cancer cells.

Based on PAP implication in cancer, an increasing number of reports have dealed with PAP modulations along the apoptotic process. They detected dephosphorylation, proteolysis and activity downregulation early in the apoptotic process in many leukemia cell lines, induced with physical agents (heat shock or nutrient deprivation) [17], or chemotherapeutic drugs (5′-fluorouracil and tamoxifen) [18–20]. In addition, PAP modulations in Daudi and Molt-3 cells treated with etoposide were found to be mutually associated with morphological evidence of apoptosis [21], whereas in human epithelioid cervix and breast cancer cell lines, treated with etoposide or cordycepin, PAP changes followed cell cycle rather than apoptosis induction [22].

In the present study, we attempted to further elucidate the revealing correlation among PAP modulations, polyadenylation and apoptosis by using the polyadenylation specific inhibitor 3′-deoxyadenosine (cordycepin). We, overall, aim at the recognition of PAP as a potential marker of cordycepin-induced apoptosis in leukemia and lymphoma cells.

Materials and methods

Cell cultures

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 cordycepin, while in exponential growth phase, for the indicated time periods. Cordycepin (Sigma Chemical Co, St. Louis, MO, USA) was prepared as a 1 mg/ml stock in distilled H_2O and stored at −40°C. The 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, St. Louis, MO, USA), incubated for 45 min at 4°C, centrifuged at 15,000×*g* for 5 min, and stored at −70°C until further use [41].

DNA fragmentation assay

Samples (10^6 intact cells/sample) were subjected to electrophoresis on 2% agarose (Sigma Chemical Co, St. Louis,

MO, USA) gel, according to Eastman protocol [42]. 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

Cordycepin (Sigma Chemical Co, St. Louis, MO, USA) 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 three d 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, St. Louis, MO, USA, 0,2 mM in PBS) and examined under a fluorescence microscope with excitation filter and LP 430 nm barrier filter [43].

MTT

Cells were assessed for sensitivity to cordycepin 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 cordycepin for the indicated time periods prior to addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co, St. Louis, MO, USA), 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 [44].

Immunoblotting and image analysis

A weight of 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 (1:2000 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 1:1000 of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co, St. Louis, MO, USA) and the immunocomplexes were visualized using 5′-bromo-4′-chloro-3′-indolyl-phosphatase (BCIP; Sigma Chemical Co, St. Louis, MO, USA) and nitro blue tetrazolium (NBT; Sigma Chemical Co, St. Louis, MO, USA) 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, St. Louis, MO, USA) [45, 46]. Protein concentration was determined by the Lowry method [47]. 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 standard assay mixture contained 200 mM Tris–HC1 (pH 8.3), 1 mM $MnC1_2$, 1 mM [^{3}H]ATP, 4 mM 2-mercaptoethanol, 1 mM poly(A) nucleotides and 40 µl of the cell extract, diluted with PBS (1×) to a final concentration of 0.2 mg of protein per ml to be assayed. The cell extract was added to the standard PAP assay mixture, as described [48], incubated at 37°C and spotted at various time intervals on GF/C discs. The enzyme reaction was stopped by soaking the filters for 5 min in 5% TCA and 1% tetrasodium pyrophosphate at 4°C. The filters were washed three times with 5% TCA at 4°C, and once with pure ethanol, and then left to dry. Radioactivity was counted in a scintillation cocktail composed of 0.3% 2,5 diphenyl-oxazol (PPO) and 0.03% 1,4-*dis*-2-(5-phenyl-oxazol) benzol (POPOP) in Toluol. The optimal conditions were obtained for saturating ATP levels (Sigma Chemical Co, St. Louis, MO, USA). One unit of PAP activity is defined as the amount of enzyme able to incorporate 1 nmol of [^{3}H]AMP (Amersham Corp.) per h at 37°C [48].

Flow cytometry

Cell cultures, 48-h old, were incubated with 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, St. Louis, MO, USA) protocol [49, 50]. According to the protocol, 10^6 cells were washed in PBS (1×) and fixed in cold 70% pure ethanol by adding it in a drop wise mode while vortexing (storage at 4°C for 30 min to 1 month). Cells were then washed in PBS (1×), centrifuged at 1,000 rpm for 5 min and the supernatant was discarded. Addition of 600 µl of the mixture followed, containing propidium iodide (50 µg/ml), Tris (10 mM), $MgCl_2$ (5 mM) and Ribonuclease A (10 µg/ml) for 30 min at room temperature and the sample was analyzed by flow cytometer.

Mitochondrial transmembrane potential ($\Delta\psi_m$)

Cell cultures, 48-h old, were incubated with 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 [51, 52].

Results

Sensitivity of Daudi and Molt-4 cells to cordycepin treatment: cell proliferation, apoptosis, necrosis, cell cycle analysis

The effect of cordycepin on two human leukemia and lymphoma cell lines (Daudi, 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 1b shows that the viability of both Daudi and Molt-4 cells was negatively affected by cordycepin in a dose- (data not shown) and time-dependent manner (since 4 and 24 h, respectively).

As MTT method assess quantitatively cell viability but cannot distinguish between growth arrest, apoptosis, or necrosis, we also analyzed cell proliferation, cell cycle arrest, DNA fragmentation and reduction in mitochondrial transmembrane potential ($\Delta\psi_m$), as well as the percentage (%) of trypan blue +ve cells (necrosis) for both of the cell lines mentioned above.

Cell growth

Cell proliferation assessment after treatment with cordycepin showed limited reduction of Daudi proliferative ability since 24 h of treatment (Fig. 1a), which could explain only in part the reduction in cell viability observed by the MTT assay (Fig. 1b). In the case of Molt-4, there was no effect on their proliferative ability (Fig. 1a).

Necrosis

Assessment of cell viability by trypan blue staining (necrosis) showed that almost no necrotic phenomena appeared, demonstrating that the variation in cell viability in both cell lines (assessed by MTT) could reflect either cell cycle arrest or apoptosis (Fig. 1c).

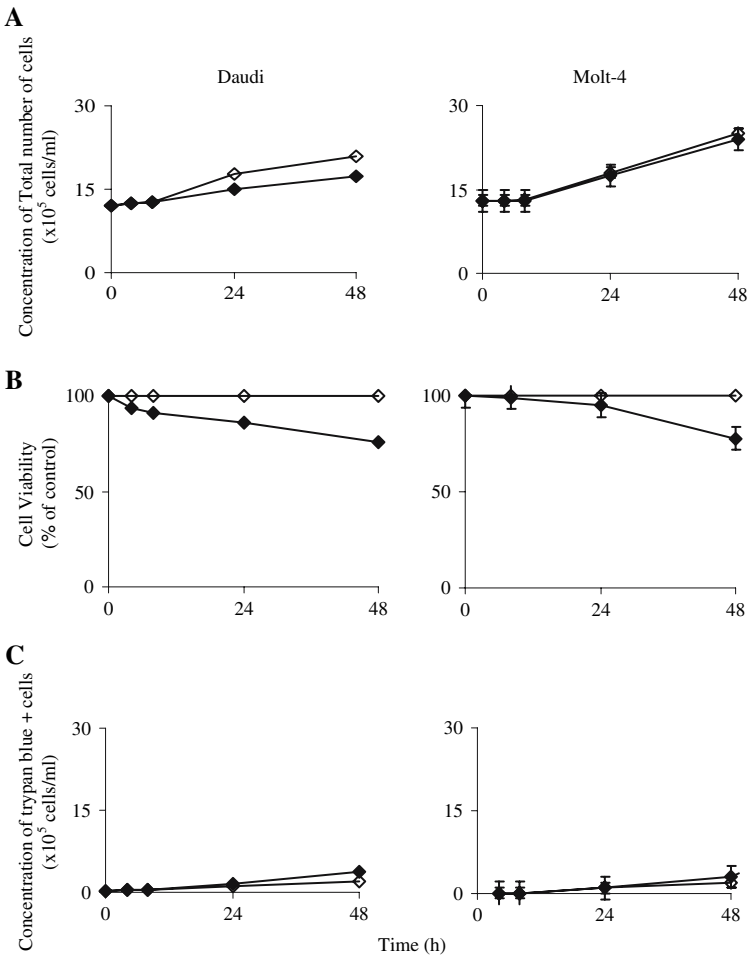

Fig. 1 Cytotoxicity of Daudi and Molt-4 cells after treatment with 0 (*open diamond*) or 20 μg/ml (*filled diamond*) of cordycepin, for 4–48 h, assessed by **a** cell proliferation, **b** MTT assay and **c** Trypan blue staining. Data points are the mean of three separate experiments

Cell cycle analysis

To further investigate the antiproliferative effect of cordycepin, we analyzed the distribution of both cell types into distinct phases of the cell cycle, after their treatment with 20 μg/ml cordycepin, by flow cytometry. Daudi cells showed limited or no cell arrest at the S phase of the cell cycle since 24 h of treatment (Table 1), whereas Molt-4 cells showed more extensive cell arrest at the G1 phase of the cell cycle since 24 h of treatment (Table 2).

DNA fragmentation

Limited morphological changes of apoptosis, such as chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies, were observed in Molt-4 cell line since 48 h and in Daudi cells since 24 h of treatment with cordycepin (Fig. 2b). Apoptosis was further assessed by the characteristic pattern of endonucleosomal DNA fragments (indicative of apoptosis). However, limited DNA fragmentation was observed (Fig. 2a), probably due to the small extent of the phenomenon, and the predominance mainly of chromatin condensation (Table 3).

Table 1 Cell cycle analysis of Daudi cells after treatment with cordycepin

Cell cycle phase	Cordycepin (μg/ml)	Percentage (%) of cells, at each time point (h)			
		4	8	24	48
G1	0	48 ± 4	49 ± 3	39 ± 3	40 ± 1
	20	47 ± 2	46 ± 1	32 ± 2	32 ± 2
S	0	46 ± 2	46 ± 1	57 ± 1	57 ± 2
	20	48 ± 3	49 ± 1	**64 ± 2**	**66 ± 1**
G2/M	0	6 ± 3	5 ± 1	4 ± 2	3 ± 1
	20	5 ± 2	5 ± 2	3 ± 1	2 ± 1

Table 2 Cell cycle analysis of Molt-4 cells after treatment with cordycepin

Cell cycle phase	Cordycepin (μg/ml)	Percentage (%) of cells, at each time point (h)			
		4	8	24	48
G1	0	51 ± 2	50 ± 1	48 ± 4	48 ± 3
	20	55 ± 1	55 ± 1	**58 ± 2**	**63 ± 1**
S	0	46 ± 2	48 ± 1	52 ± 3	51 ± 2
	20	40 ± 3	37 ± 1	41 ± 2	36 ± 1
G2/M	0	3 ± 1	2 ± 1	0 ± 0	1 ± 1
	20	5 ± 1	8 ± 2	1 ± 1	1 ± 2

$\Delta\psi_m$

We also assessed changes in the mitochondrial transmembrane potential ($\Delta\psi_m$), an early marker of mitochondrial apoptotic pathway in order to distinguish possible apoptotic pathways induced by cordycepin. The results showed that $\Delta\psi_m$ was almost unaffected in Molt-4 cells (Fig. 1a), whereas in Daudi cells it was highly reduced (Fig. 1a).

Taken together, the results presented above demonstrate that both cell lines examined are induced to apoptosis with 20 μg/ml cordycepin and therefore the observed variation in cell viability reflected mainly apoptosis. However, apoptosis was induced probably via distinct pathways in the two cell lines under study, which was mitochondrial in the case of Daudi and non-mitochondrial in the case of Molt-4 cells. In addition, the two cell lines showed distinct sensitivity to the apoptotic, antiproliferative and cytotoxic effect of the agent.

PAP modulations in cordycepin-treated Daudi and Molt-4 cells

Treatment of Daudi cells with 20 μg/ml cordycepin for the indicated time periods (Fig. 3) did not affect significantly either PAP isoforms or activity levels until 48 h. However, in the case of Molt-4 cells, all three PAP phosphorylated isoforms (100, 106 and 80 kDa) are significantly increased at 8 h of treatment, as well as PAP activity levels, and to a lesser extent at 24 and 48 h of treatment, whereas at 48 h there is a smaller increase in the 106 kDa isoform of PAP and a decrease in the other two PAP isoforms.

Discussion

Lately, analysis of the expression profiles of an increasing number of genes involved in cancer development, progression and therapeutics, has revealed that many of them are implicated in cancer cell apoptosis. Such an example is the *PAP* and the *BCL2* gene family members, as well as many more [53, 54]. Both, the BCL2 and the PAP family of proteins (including *BCL2L12*, a new member) play an important regulatory role in the apoptotic process, showing prognostic potential and possessing an important role in determining response to chemotherapy in distinct types of cancer, as well as in cancer cell systems [53–57].

Our present research approach, aim at the recognition of PAP as a potential marker of apoptosis in cancer therapy outcome. The specific tissue expression and activity patterns of PAP isoforms in distinct types of cancer suggest multiple physiological roles. However, much need to be learned about the expression and activity of PAP isoforms in diverse cancer types and the underlying biological effects resulting from their enzymatic action.

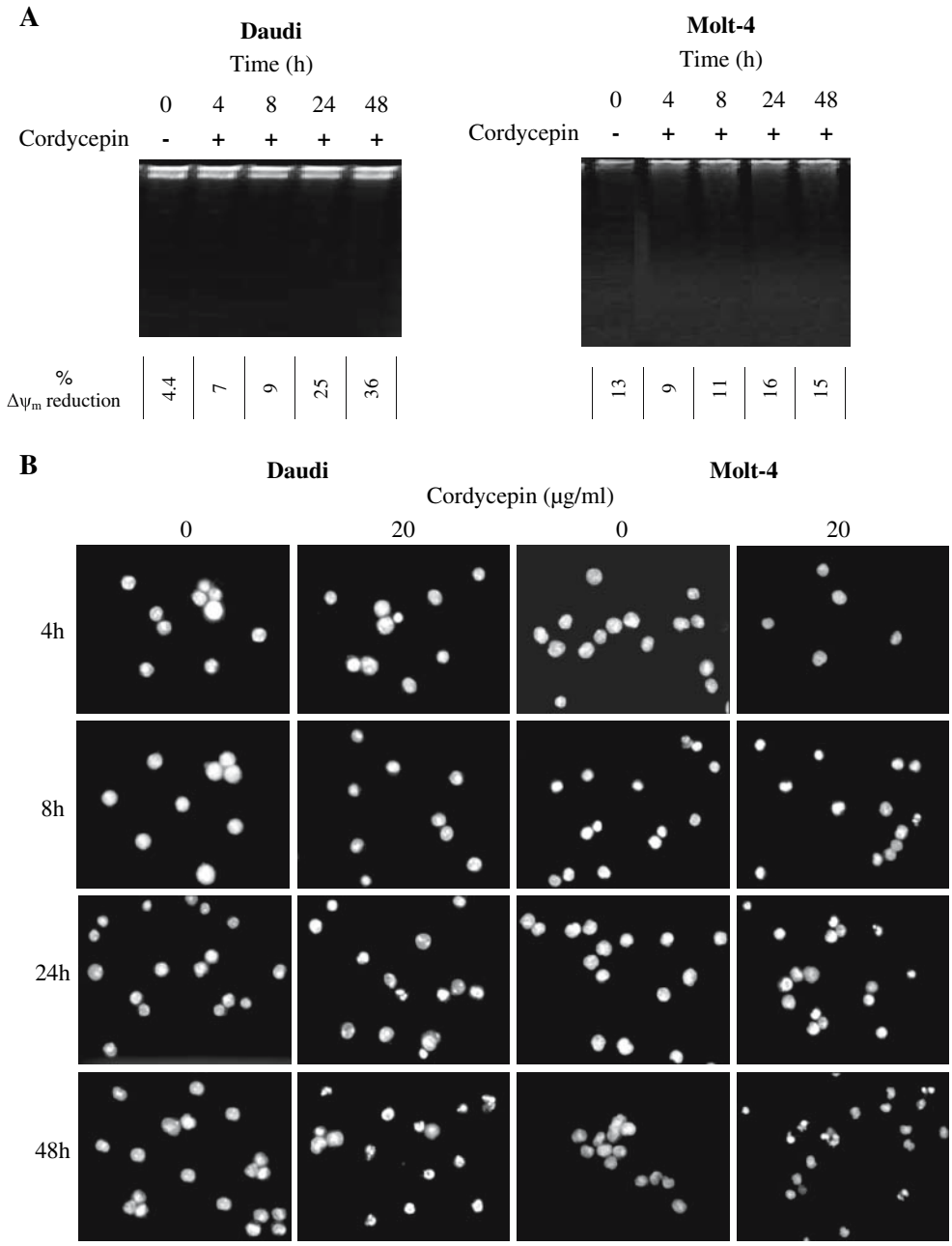

Fig. 2 **a** DNA cleavage, % of reduction in mitochondrial transmembrane potential and **b** DAPI staining (magnification, ×400), of Daudi and Molt-4 cells, in the absence or presence of 20 μg/ml of cordycepin, for the indicated time periods

Cordycepin is a well-known polyadenylation inhibitor and an analogue of adenosine, which is used in the treatment of many types of leukemia [4–6, 11, 13]. Until a few years ago, it was believed that the use of adenosine analogues in chemotherapeutic regimens was mainly based on their antiproliferative and cytotoxic effect. However, recent studies have shown that purine analogs have demonstrated promise for cancer treatment and more specifically, many adenosine analogues demonstrate their anticancer activity through entering cellular nucleotide pools and induction of differentiation [2, 3] or programmed cell death [5–9], processes which in many cases, are cell type-dependent. Therefore, it was expected that inhibition of polyadenylation with cordycepin would result in intervention in PAP levels and consequently changes in the apoptotic process.

In the present study, we intervened in the polyadenylation process by studying the effect of cordycepin (a polyadenylation inhibitor) on Daudi and Molt-4 cell lines, as well as PAP activity and isoforms. Our results showed that PAP activity and isoforms are modulated as a response to

Table 3 PAP form modulations along treatment of Daudi and Molt-4 leukemia cells with cordycepin

PAP form molecular weight (kDa)	Time of exposure (h) to 20 μg/ml cordycepin			
	4	8	24	48
Daudi				
106	4	13	2	1
100	20	21	−7	−2
80	7	14	21	11
Molt-4				
106	10.6	60.4	50	24
100	0.22	25.8	4.4	−36
80	−8.5	26.8	10.7	−36

Changes based on quantitative densitometric data, according to western blot analysis performed using the NIH Image program, developed at the U.S. National Institute of Health (http://www.rsb.info.nih.gov/nih-image/). % changes = $B - A/A \times 100$, where A is the densitometric data for the control sample and B that for the sample treated with drug. A positive number indicates an increase and a negative number a decrease in the level of PAP forms expression

cell treatment with cordycepin, only in Molt-4 cells and not in Daudi cells. Inhibition of polyadenylation with cordycepin causes chromatin condensation and whole cell shrinkage, without endonucleosomal cleavage of DNA, in both cell lines, although with delay and to a greater extent in Molt-4 cells, and with decrease of $\Delta\psi_m$ appearing only in Daudi cells. Therefore, treatment of both cell lines with cordycepin leads to apoptosis induction, probably through distinct pathways (mitochondrial in the case of Daudi and non-mitochondrial in the case of Molt-4), which may account for the distinct cell response as far as PAP activity and isoforms is concerned. Chromatin condensation and fragmentation observed in Molt-4 cells may be due to the inhibitory action of cordycepin on TdT, which has as a result the induction of cytotoxic and apoptotic phenomena in cases of TdT^{+ve} cells, such as Molt-4 [5]. Apoptosis induction by an adenosine derivative, 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-*N*-methyl-beta-D-ribofuranonamide (Cl-IB-MECA), in Molt-4 cells, has also been previously reported [58].

In the case of Molt-4 cells, PAP activity and isoforms increase, which occurs as a response to cordycepin treatment, appears prior to any characteristic apoptotic or cytotoxic changes, along the early reversible phase of cell commitment to apoptosis. This pattern of PAP modulations may imply that the cellular need for polyadenylation, in order for non-mitochondrial apoptosis to be induced via a polyadenylation specific inhibitor, may be expressed by increase of PAP enzyme activity and isoforms levels prior to any morphological changes of apoptosis, so that specific mRNAs are stabilized. However, as the apoptotic phenomenon progresses, PAP isoforms increase remain, but it progressively becomes eliminated, leading finally to the decrease in PAP protein levels.

An alternative approach in the explanation of our findings concerns the investigation of a possible correlation among PAP modulations and the course of cell cycle in the two cell lines. We have shown that only in the Molt-4 cell line, cordycepin treatment causes cell accumulation at the G1 phase of the cell cycle, in parallel with the appearance of changes in chromatin structure (condensation and/or

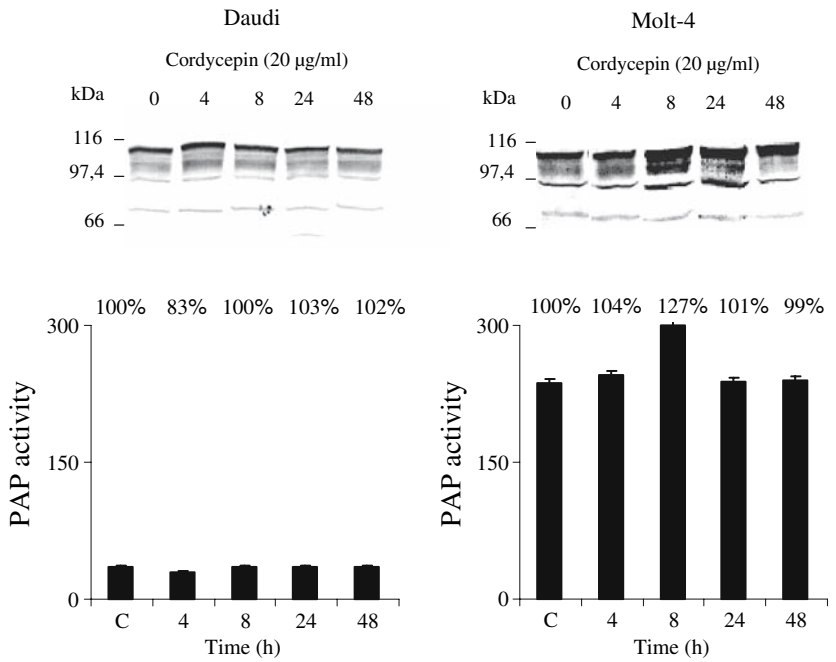

Fig. 3 Activity and western blot analysis of PAP enzyme forms in lysates from Daudi and Molt-4 cells in the absence or presence of 20 μg/ml cordycepin, for the indicated time periods. The numbers (%) represent the percentage of control PAP activity after treatment with cordycepin for the indicated time periods

cleavage). However, in Daudi cells, there is no significant cell arrest, with PAP forms and activity remaining unchanged. This implies that Molt-4 cells arrest at the first stage of DNA repair mechanisms (G1 phase) and advance in the production of mRNAs which will be expressed for the execution of DNA synthesis (S phase). PAP is therefore necessary to be present within Molt-4 cells in a continuous active form, explaining the increased PAP activity and isoform levels that were observed. What is more, the role of the distinct origin of the two cell lines under study, in their differential response to drug treatment, should not be excluded. This in conjunction with the fact that Daudi cells show much lower PAP activity levels, in comparison to Molt-4 cells, may imply that since T cells (Molt-4) possess a more comprehensive role within the cells than B cells (Daudi), they may be more metabolically active, as well as their enzymes involved in nucleotide metabolism, such as PAP, and therefore they may respond more rapidly and markedly to exogenous stress.

Overall, we could say that the differentiated modulations of PAP in the two leukaemia and lymphoma cell lines may be a result of the additive effect of the changes in cell cycle and apoptotic pathway induced.

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References

1. Chen LS, Sheppard TL (2004) Chain termination and inhibition of Saccharomyces cerevisiae poly(A) polymerase by C-8-modified ATP analogs. J Biol Chem 279:40405–40411
2. Honma Y, Niitsu N (2000) Vidarabine and 2-deoxycoformycin as antileukemic agents against monocytic leukemia. Leuk Lymphoma 39:57–66
3. Levenson R, Kernen J, Housman D (1979) Synchronization of MEL cell commitment with cordycepin. Cell 18:1073–1078
4. Niitsu N, Umeda M, Honma Y (2000) Myeloid and monocytoid leukemia cells have different sensitivity to differentiation-inducing activity of deoxyadenosine analogs. Leuk Res 24:1–9
5. Koc Y, Urbano AG, Sweeney EB, McCaffrey R (1996) Induction of apoptosis by cordycepin in ADA-inhibited TdT-positive leukemia cells. Leukemia 10:1019–1024
6. Niitsu N, Yamaguchi Y, Umeda M, Honma Y (1998) Human monocytoid leukemia cells are highly sensitive to apoptosis induced by 2'-deoxycoformycin and 2'- deoxyadenosine: association with dATP-dependent activation of caspase-3. Blood 92:3368–3375
7. Wu JY, Zhang QX, Leung PH (2007) Inhibitory effects of ethyl acetate extract of Cordyceps sinensis mycelium on various cancer cells in culture and B16 melanoma in C57BL/6 mice. Phytomedicine 14:43–49
8. Wu WC, Hsiao JR, Lian YY, Lin CY, Huang BM (2007) The apoptotic effect of cordycepin on human OEC-M1 oral cancer cell line. Cancer Chemother Pharmacol 60:103–111
9. Wang BJ, Won SJ, Yu ZR, Su CL (2005) Free radical scavenging and apoptotic effects of Cordyceps sinensis fractionated by supercritical carbon dioxide. Food Chem Toxicol 43:543–552
10. Cunningham KG, Hutchinson SA, Manson W, Spring FS (1950) Cordycepin, a metabolic product isolated from cultures of Cordyceps militaris. Nature 166:949
11. Müller WE, Seibert G, Beyer R, Breter HJ, Maidhof A, Zahn RK (1977) Effect of cordycepin on nucleic acid metabolism in L5178Y cells and on nucleic acid -synthesizing enzyme systems. Cancer Res 37:3824–3833
12. Piga A, Ganeshaguru K, Green ES, Sheridan B, Hoffbrand AV (1989) Selective toxicity of purine nucleosides to human leukaemic cells. Adv Exp Med Biol 253B:291–298
13. Kodama EN, McCaffrey RP, Yusa K, Mitsuya H (2000) Antileukemic activity and mechanism of action of cordycepin against terminal deoxynucleotidyl transferasepositive (TdT+) leukemic cells. Biochem Pharmacol 59:273–281
14. Nakamura K, Yoshikawa N, Yamaguchi Y, Kagota S, Shinozuka K, Kunitomo M (2006) Antitumor effect of cordycepin (3'-deoxyadenosine) on mouse melanoma and lung carcinoma cells involves adenosine A3 receptor stimulation. Anticancer Res 26:43–47
15. Nakamura K, Konoha K, Yoshikawa N, Yamaguchi Y, Kagota S, Shinozuka K, Kunitomo M (2005) Effect of cordycepin (3'-deoxyadenosine) on hematogenic lung metastatic model mice. In Vivo 19:137–141
16. Foss FM (2000) Combination therapy with purine nucleoside analogs. Oncology (Williston Park) 14:31–35
17. Atabasides H, Tsiapalis CM, Havredaki M (1998) Dephosphorylation, proteolysis and reduced activity of poly(A) polymerase associated with U937 cell apoptosis. Exp Cell Res 244:433–440
18. Balatsos NA, Havredaki M, Tsiapalis CM (2000a) Anticancer drug action on poly(A) polymerase activity and isoforms during HeLa and WISH cell apoptosis. Int J Biol Markers 15:171–178
19. Balatsos NA, Havredaki M, Tsiapalis CM (2000b) Early 5-fluorouracil-induced changes of poly(A) polymerase in HeLa and WISH cells. Int J Biol Markers 15:294–299
20. Balatsos NA, Lallas G, Havredaki M, Tsiapalis CM (2001) Drug action on poly(A) polymerase activity and isoforms during U937 cell apoptosis. J Exp Clin Cancer Res 20:63–69
21. Thomadaki E, Havredaki M, Tsiapalis CM (2004) PAP modulations in Daudi cells and Molt-3 cells treated with etoposide are mutually associated with morphological evidence of apoptosis. Int J Biol Markers 19:203–212
22. Thomadaki H, Tsiapalis CM, Scorilas A (2005) Polyadenylate polymerase modulations in human epithelioid cervix and breast cancer cell lines, treated with etoposide or cordycepin, follow cell cycle rather than apoptosis induction. Biol Chem 386:471–480
23. Wahle E, Martin G, Schiltz E, Keller W (1991) Isolation and expression of cDNA clones encoding mammalian poly(A) polymerase. EMBO J 10:4251–4257
24. Zhao W, Manley JL (1996) Complex alternative RNA processing generates an unexpected diversity of poly(A) polymerase isoforms. Mol Cell Biol 16:2378–2386
25. Kashiwabara S, Zhuang T, Yamagata K, Noguchi J, Fukamizu A, Baba T (2000) Identification of a novel isoform of poly(A) polymerase, TPAP, specifically present in the cytoplasm of spermatogenic cells. Dev Biol 228:106–115
26. Topalian SL, Kaneko S, Gonzales MI, Bond GL, Ward Y, Manley JL (2001) Identification and functional characterization of neo-poly(A) polymerase, an RNA processing enzyme overexpressed in human tumors. Mol Cell Biol 21:5614–5623
27. Kyriakopoulou CB, Nordvarg H, Virtanen A (2001) A novel nuclear human poly(A) polymerase (PAP), PAP gamma. J Biol Chem 276:33504–33511

28. Scorilas A (2002) Polyadenylate polymerase (PAP) and 3' end pre-mRNA processing: function, assays, and association with disease. Crit Rev Clin Lab Sci J39:193–224
29. Wahle E, Ruegsegger U (1999) 3'-end processing of pre-mRNA in eukaryotes. FEMS Microbiol Rev 23:277–295
30. Raabe T, Bollum FJ, Manley JL (1991) Primary structure and expression of bovine poly(A) polymerase. Nature 353:229–234
31. Zhao W, Manley JL (1998) Deregulation of poly(A) polymerase interferes with cell growth. Mol Cell Biol 18:5010–5020
32. Kazazoglou T, Tsiapalis CM, Havredaki M (1987) Polyadenylate polymerase activity in stationary and growing cell cultures. Exp Cell Biol 55:164–172
33. Ballantyne S, Bilger A, Astrom J, Virtanen A, Wickens M (1995) Poly(A) polymerases in the nucleus and cytoplasm of frog oocytes: dynamic changes during oocyte maturation and early development. RNA 1:64–78
34. Perez S, Trangas T, Kokkinopoulos D, Tsiapalis CM, Papamichail M (1987) Polyadenylic acid metabolizing enzyme levels during induction of differentiation in a human leukemia T-cell line with phorbol ester. J Natl Cancer Inst 78:407–411
35. Trangas T, Courtis N, Pangalis GA, Cosmides HV, Ioannides C, Papamichail M, Tsiapalis CM (1984) Polyadenylic acid polymerase activity in normal and leukemic human leukocytes. Cancer Res 44:3691–3697
36. Pangalis GA, Trangas T, Roussou PA, Tsiapalis CM (1985) Poly(A)-polymerase activity in chronic lymphocytic leukemia of the B cell type. Acta Haematol 74:31–34
37. Sasaki R, Minowada J, Bollum FJ, Miura Y (1990) Polyadenylic acid polymerase activity in chronic myelogenous leukemia. Leuk Res 14:273–278
38. Scorilas A, Courtis N, Yotis J, Talieri M, Michailakis M, Trangas T (1998) Poly(A) polymerase activity levels in breast tumour cytosols. J Exp Clin Cancer Res 17:511–518
39. Scorilas A, Talieri M, Ardavanis A, Courtis N, Yotis J, Dimitriadis E, Tsiapalis C, Trangas T (2000) Polyadenylate polymerase enzymatic activity in mammary tumor cytosols: a new independent prognostic marker in primary breast cancer. Cancer Res 60:5427–5433
40. Bodeker H, Vasseur S, Dusetti NJ, Dagorn JC, Iovanna JL (1998) PAP gene transcription induced by cycloheximide in AR4-2J cells involves ADP-ribosylation. Biochem Biophys Res Commun 251:710–713
41. Casciola-Rosen LA, Miller DK, Anhalt GJ, Rosen A (1994) Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. J Biol Chem 269:30757–30760
42. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40:2353–2362
43. Lazebnik YA, Cole S, Cooke CA, Nelson WG, Earnshaw WC (1993) Nuclear events of apoptosis in cell – free mitotic extracts: a model system for analysis of the active phase of apoptosis. J Cell Biol 123:7–22
44. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
45. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
46. Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate—polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195–203
47. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
48. Bollum FJ, Chang LM, Tsiapalis CM, Dorson JW (1974) Nucleotide polymerizing enzymes from calf thymus gland. In: Colowick SP, Kaplan NO (eds) Methods enzymol, vol 29. Academic, New York, pp 70
49. Didelot C, Mirjolet JF, Barberi-Heyob M, Ramacci C, Merlin JL (2002) Radiation could induce p53-independent and cell cycle-unrelated apoptosis in 5-fluorouracil radiosensitized head and neck carcinoma cells. Can J Physiol Pharmacol 80:638–643
50. Chou RH, Huang H (2002) Restoration of p53 tumor suppressor pathway in human cervical carcinoma cells by sodium arsenite. Biochem Biophys Res Commun 293:298–306
51. Polla BS, Kantengwa S, Francois D, Salvioli S, Franceschi C, Marsac C, Cossarizza A (1996) Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. Proc Natl Acad Sci USA 93:6458–6463
52. Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A (1997) JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\psi_m$ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 411:77–82
53. Thomadaki H, Scorilas A (2006) BCL2 family of apoptosis-related genes: functions and clinical implications in cancer. Crit Rev Clin Lab Sci 43:1–67
54. Thomadaki H, Talieri M, Scorilas A (2006) Prognostic value of the apoptosis related genes BCL2 and BCL2L12 in breast cancer. Cancer Lett (in press)
55. Floros KV, Thomadaki H, Lallas G, Katsaros N, Talieri M, Scorilas A (2003) Cisplatin-induced apoptosis in HL-60 human promyelocytic leukemia cells: differential expression of BCL2 and novel apoptosis-related gene BCL2L12. Ann N Y Acad Sci 1010:153–158
56. Floros KV, Thomadaki H, Katsaros N, Talieri M, Scorilas A (2004) mRNA expression analysis of a variety of apoptosis-related genes, including the novel gene of the BCL2-family, BCL2L12, in HL-60 leukemia cells after treatment with carboplatin and doxorubicin. Biol Chem 385:1099–1103
57. Thomadaki H, Talieri M, Scorilas A (2006) Treatment of MCF-7 cells with taxol and etoposide induces distinct alterations in the expression of apoptosis-related genes BCL2, BCL2L12, BAX, CASPASE-9 and FAS. Biol Chem 387:1081–1086
58. Kim SG, Ravi G, Hoffmann C, Jung YJ, Kim M, Chen A, Jacobson KA (2002) p53-Independent induction of Fas and apoptosis in leukemic cells by an adenosine derivative, Cl-IB-MECA. Biochem Pharmacol 63:871–880