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ORIGINAL ARTICLE

Growth inhibitory activity of a novel lectin from *Cliona varians* against K562 human erythroleukemia cells

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Abstract
Purpose In this study, the antitumoral potential of a novel lectin (CvL) purified from the marine sponge *Cliona varians* was studied in different cancer cell lines.
Methods CvL cytotoxicity was evaluated in mammalian tumor cells and in normal human peripheral blood lymphocytes by the MTT assay using the same range of concentrations (1–150 μg ml^{-1}). The mechanisms involved in K562 cell death were investigated by confocal fluorescence microscopy, flow cytometry and immunoblot.
Results CvL inhibited the growth of human leukemia cells, with IC_{50} values of 70 and 100 μg ml^{-1} for K562 and JURKAT cells, respectively, but it was ineffective on blood lymphocytes and solid tumor cell lines. K562 cell death occurred 72 h after exposure to the lectin and with signs of apoptosis, as analyzed by DAPI and annexin V/PI staining. Investigation of the possible mediators of this process showed that cell death occurred via a caspase-independent pathway. Confocal fluorescence microscopy indicated a pivotal role for the lysosomal protease cathepsin B in mediating cell death. Accordingly, pre-incubation of K562 cells with the cathepsin inhibitor L-trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) abolished CvL cytotoxic effect. Furthermore, we found upregulation of tumor necrosis factor receptor 1 (TNFR1) and down-modulation of p65 subunit of nuclear factor kappa B (NFκB) expression in CvL-treated cells. These effects were accompanied by increased levels of p21 and reduced expression of pRb, suggesting that CvL can induce cell cycle arrest.
Conclusions Collectively, these findings indicate an anti-leukemic effect for CvL and suggest that cathepsin B acts as a death mediator in CvL-induced cytotoxicity possibly in an uncharacterized connection with the membrane death receptor pathway.

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Keywords Lectin · *Cliona varians* · K562 · Apoptosis · Cathepsin B · Cytotoxicity · Antitumor

Introduction

Successful treatment of cancer with chemotherapy is largely dependent on its ability to trigger cell death in tumor cells. Many cytotoxic agents produce cell death by inducing apoptosis. Although most current studies about anticancer drugs focus on apoptosis induction by triggering caspases, mitochondrial energy changes and plasma membrane-bound death receptors, evidence has been brought forward for a role of lysosomes in the cytotoxic effects of several chemotherapeutic agents and cytokines. In particular, cathepsins B and D released from the lysosomes into the cytosol have been implicated as effector proteases in the cascade of cell death [1–3].

Lectins are carbohydrate-binding proteins that occur ubiquitously in nature [4]. Biological properties of lectins include cell agglutination, mitogenic activity, antibacterial and antitumoral effects [5–9]. In addition, several lectins have been used to differentiate malignant from benign tumors and the degree of glycosylation associated with metastasis [10–12]. Furthermore, some lectins have been shown to induce apoptosis in different cell lines, thus explaining their cytotoxicity [13–16].

In recent years, bioactive compounds isolated from marine sources have gained attention due to their potential application as pharmacological tools in the diagnosis field. It has been shown that lectins can be isolated from the hemolymph and cells of many marine invertebrates [17–20]. Recently, the ability of *Eucheuma serra* agglutinin (ESA), which is a lectin derived from the marine red alga *E. serra*, to induce cell death was demonstrated in vitro and in vivo [21]. We have recently purified and characterized a lectin from the marine sponge *Cliona varians*, which presents Ca^{2+} dependent hemagglutinating activity towards papainized type A erythrocytes [22]. This lectin, termed CvL, is a tetrameric glycoprotein of 28-kDa subunits linked by disulphide bridges with a molecular mass of 106 kDa, with preferential binding properties to D-galactose. This lectin also showed antibacterial effects against pathogenic gram positive bacteria and agglutinating activity towards *Leishmania chagasi* promastigotes, suggesting a potential pharmacological application. Few reports exist on the induction of apoptosis in cancer cells by lectins isolated from marine sources. In this work, we report that CvL causes leukemia (K562 and JURKAT) cell killing but is ineffective on normal human peripheral blood lymphocytes and solid tumor cell lines. The response induced by CvL is clearly different from that mediated primarily via caspases. Investigation of the possible effectors of this process indicates that K562 cell death occurs even in the presence of Bcl-2 family proteins and in the absence of caspase activation, suggesting that pathways that form the basic apoptotic machinery in cells played only a minor role in triggering CvL-induced cell death. Nevertheless, the sequence of events induced by CvL in K562 cells involves upregulation of tumor necrosis factor receptor 1 (TNFR1) and down-modulation of nuclear factor kappa B (NFκB) p65 subunit expression. These effects are accompanied by increased levels of p21 and reduced expression of pRb, suggesting that CvL is capable of both apoptosis induction and cell cycle arrest. In addition, two lines of evidence suggest a central role for the lysosomal protease cathepsin B in mediating cell death. First, pre-incubation of cells with cathepsin inhibitor E-64 results in a strong protection against CvL-induced K562 cell death. Second, CvL triggers release of cathepsin B as evidenced by fluorescence microscopy. On the basis of these findings, we propose that CvL kills K562 cells mainly via a caspase-independent mechanism, possibly involving the death receptor pathway. Yet, these results point to a proximal role for cathepsin B in this death pathway.

Materials and methods

Reagents

Antibodies against tumor necrosis factor (TNF) receptor 1 (TNFR1), Bax, Bcl-2, p21 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against nuclear factor kappa B (NFκB) p65 and phospho-retinoblastoma protein (pRb), cleaved caspase-3 (Asp175) antibody conjugated to Alexa Fluor 488 and anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit IgG Alexa Fluor 488-conjugated antibody, and 4′-6′-diamidino-2-phenylindole (DAPI) dihydrochloride were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Caspase-3, caspase-8 and caspase-9 colorimetric assay kits, and the polyclonal antibody against cathepsin B were obtained from R&D Systems (Minneapolis, MN, USA). Paraformaldehyde and Fluoromount-G were from Electron Microscopy Sciences (Hatfield, PA, USA), and Cell-Tak (cell and tissue adhesive) was from Collaborative Biomedical Products (Bedford, MA, USA). Annexin V conjugated to fluorescein isothiocyanate (FITC) and propidium iodide were from BD Biosciences (CA, USA). The cysteine protease inhibitor, L-trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), RNase type I and phytohemagglutinin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of CvL

Methodology developed by Moura et al. [22] was used to purify CvL from *Cliona varians* marine sponge. The

hemagglutinating activity of CvL was assayed in papainized treated human erythrocytes [23] and the sugar specificity of the lectin was determined as described previously [22]. All inhibitors to be tested were dissolved in 150 mM NaCl at an initial concentration of 200 mM for monosaccharides (galactose, glucose, fucose, ribose, *N*-acetyl-glycosamine and glucuronic acid) and disaccharides (maltose, sucrose and lactose). SDS polyacrylamide (12%) gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli [24].

Cell lines and culture

The K562 erythroleukemia cell line and the immature T-cell line JURKAT, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in suspensions in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS, Sigma Chemical Co.), 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin in a humidified atmosphere at 37°C in 5% CO_2. Cells were seeded (2×10^4 cells ml^{-1}) in 96-well microtiter plates and incubated with different concentrations of CvL for 72 h. To inhibit proteolytic activity, K562 cells (2×10^4 cells ml^{-1}) were incubated with 5 μM of the irreversible cysteine protease inhibitor E-64 for 2 h before a 72-h treatment with different concentrations of CvL in a humidified atmosphere at 37°C in 5% CO_2. Adherent tumor cells (B16 murine melanoma, human renal carcinoma cell line 786-O and human androgen-independent prostate cancer cell line PC3), obtained from ATCC, were grown in RPMI 1640 medium (B16 melanoma and PC3 cells) or DMEM (Dulbecco's modified Eagle's medium, Sigma Chemical Co., 786-O cells) supplemented with 10% FCS, 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin in a humidified atmosphere at 37°C in 5% CO_2. Cells were incubated in 24-well plates until reach the semiconfluence and then treated with different concentrations of CvL for 24 h.

Leukocyte culture

Peripheral human blood was obtained by venipuncture from healthy adult donors, diluted with an equal volume of RPMI 1640 medium, then layered over Ficoll-Hypaque density gradient separation solution (1.077 g ml^{-1}), and centrifuged at 400 *g* for 20 min at room temperature. The mononuclear cell layer was removed, washed twice in RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 2 mM glutamine (Sigma Chemical Co.), antibiotics and 10% FCS. Leukocytes at a density of 1×10^6 plating ml^{-1} were cultured with 5 μg ml^{-1} of phytohemagglutinin in 96-well microtiter plates, in the presence and absence of CvL, for 72 h in a humidified atmosphere at 37°C in 5% CO_2.

Cell proliferation and viability assays

Cell proliferation was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co.) reduction test, and the trypan blue dye exclusion assay was used to assess cell viability as previously described [25]. Each concentration was tested in three different experiments run in four replicates. CvL-treated cell proliferation and viability were expressed as percent of the cell proliferation or viability of untreated control cells (100%).

Apoptosis index

Nuclear changes indicative of apoptosis (i.e., chromatin condensation and nuclear fragmentation) were assessed using the DNA binding dye 4′–6′-diamidino-2-phenylindole (DAPI) dihydrochloride [26, 27]. After treatment with CvL, cells were washed in ice-cold phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde in ice-cold PBS for 30 min and permeabilized in PBS containing 0.1% saponin and 1% bovine serum albumin (BSA) for 10 min. After washing in PBS, cells were incubated with DAPI (1 μg ml^{-1}) for 30 min at room temperature in the dark. After incubation, cells were placed onto glass coverslips covered with Cell-Tak, washed in PBS and mounted in Fluoromount-G. Cells were viewed by fluorescence microscopy (Leica DMIRBE Microscope, Benshein, Germany) using the blue fluorescence filter (330–380 nm). The apoptotic index was determined by counting 100 cells per field of view in 3 different fields per sample. Each sample was done in duplicate and the number of apoptotic cells was expressed as a percentage of the total number of cells.

Caspase activity

K562 cells were treated with CvL as indicated and analysis of the activation of caspase-3, caspase-8 and caspase-9 was performed using colorimetric protease kits according to the manufacturer's recommendations. The activity of caspases was determined by the measurement at 405 nm of *p*-nitroanaline (*p*NA) released from the cleavage of caspase-3 (DEVD-*p*NA), caspase-8 (IETD-*p*NA) and caspase-9 (LEHD-*p*NA) substrates. The enzyme activities were calculated in pmol min^{-1} (mg protein)$^{-1}$ (extinction coefficient of *p*NA was 10,000 M^{-1} cm^{-1}) and expressed relative to controls. In the absence of CvL, the activity was considered to be 100%. Caspase-3 activation was also

evaluated by flow cytometric analysis of endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 according to the manufacturer's instructions (Cell Signaling). After treatment, K562 cells were harvested by centrifugation, washed with cold PBS and fixed in paraformaldehyde 2% in PBS (v/v) for 30 min. Cells were then permeabilized in PBS containing 0.01% saponin for 15 min and blocked in PBS containing 1% BSA (PBS/1% BSA) for 30 min at room temperature. Afterwards, 10 µl of cleaved caspase-3 (Asp175) Alexa Fluor 488-conjugated antibody were added and cells were incubated in the dark at room temperature for 1 h. After washing in PBS/1% BSA, cells were resuspended in 400 µL PBS and analyzed (10,000 events were collected per sample) in a FACSCalibur flow cytometer (Beckton Dickinson, CA, USA) using the CellQuest software. Control cells were treated with medium only.

Flow cytometric analysis of cell death after propidium iodide staining

Cells were seeded in 6-well plates and cultured in the presence of 10% FBS medium for 24 h. After 24 h of culture in serum free RPMI for cell cycle synchronization, cells were treated with CvL for 72 h. After this period, cells were harvested, washed with cold PBS and fixed and permeabilized as described previously. Then, cells were treated with 4 µg ml^{-1} RNase type I for 1 h at 37°C and resuspended in PBS. Cells were stained with 25 µg ml^{-1} propidium iodide (PI) and analyzed (10,000 events were collected per sample) in a FACSCalibur flow cytometer using the CellQuest software. The DNA content was evaluated using a FL2H detector in a logarithmic scale. The analysis of cell percentage in the sub-G_1 phase of cell cycle was performed using the CellQuest software. Control cells were treated with medium only.

Annexin V-FITC/PI double-staining and analysis by flow cytometry

After treatment with CvL as described above, K562 cells were harvested washed with cold PBS and resuspended in binding buffer (0.01 M Hepes, pH 7.4, 0.14 M NaCl and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells ml^{-1}. The suspensions were transferred to 5-ml tubes and 5 µL Annexin V-FITC and 5 µg ml^{-1} PI were added. The cells were incubated at room temperature for 20 min, after which 300 µL binding buffer was added and analysis (10,000 events were collected per sample) was performed in a FACSCalibur flow cytometer using the CellQuest software. Control cells were treated with medium only.

Immunofluorescence staining of cathepsin B and microscopic analysis of cells

Immunoassays were performed in K562 cells treated with vehicle and CvL as described above. Cells were fixed with 2% paraformaldehyde in ice-cold PBS for 30 min and permeabilized in PBS containing 0.1% saponin and 1% BSA for 10 min. After washing in PBS, cells were incubated for 1 h with anti-cathepsin B polyclonal antibody at 1:50 dilution, and then labeled with anti-rabbit IgG Alexa Fluor 488-conjugated antibody (1:300 dilution) for 30 min at room temperature in the dark. For imaging, cells were placed onto glass coverslips covered with Cell-Tak, washed in PBS and mounted in Fluoromount-G. Cells were then analyzed using an inverted laser scanning confocal microscope (Zeiss LSM 510 Confocal Microscope, Jena, Germany). The sample was excited with argon laser 488 nm and emission detected using a bypass filter at 500–550 nm.

Immunoblot analysis

K562 cells (3×10^7) were lysed in 200 µl lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors (1 µg ml^{-1} aprotinin, 10 µg ml^{-1} leupeptin and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride)] for 2 h in ice. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Lowry protein assay [28]. An equal volume of 2× sodium dodecyl sulphate (SDS) gel loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue and 20% glycerol] was added to samples, which were subsequently boiled for 10 min. Equal quantities of protein (50 µg) were loaded onto SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 1% fat-free dried milk or 2% BSA in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) and incubated overnight at 4°C with appropriate primary antibody at 1:1,000 dilution. After washing in TBST, membranes were incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies, at 1:2,000 dilutions, in blocking buffer for 1 h. Detection was performed using enhanced chemiluminescence.

Statistical analysis

All data represent at least three independent experiments and are expressed as mean ± standard deviation unless otherwise indicated. Differences between groups were compared using analysis of variance (ANOVA) for repeated measures and a post hoc Tukey test to correct for multiple

comparisons. Differences were considered significant when P value was less than 0.05.

Results

Effects of CvL on cell proliferation and viability of tumor cells and normal lymphocytes

The cytotoxicity of CvL to human leukemia cells was investigated after an incubation period of 72 h using the colorimetric MTT assay. As shown in Fig. 1a, K562 cell proliferation was inhibited in a dose-dependent manner in response to increasing concentrations of CvL (1–150 μg ml^{-1}). The 50% of inhibition (IC_{50} value) was obtained with a concentration around 70 μg ml^{-1} of CvL. Effects of the lectin on K562 cell viability was verified using the trypan blue dye exclusion method. At the concentration of 20 μg ml^{-1} CvL, it revealed 80% of cell viability as long as the maximum decrease (25%) was seen after treatment with 80 μg ml^{-1} (Fig. 1b). Even though CvL was also able to inhibit the proliferation of human immature T cells (JURKAT cell line), these cells were less sensitive to the lectin, presenting an IC_{50} value 100 μg ml^{-1} (Fig. 1a). In contrast, no cytotoxicity to normal proliferating lymphocytes from human peripheral blood was observed after incubation with the lectin in the same range of concentrations, as verified by the MTT assay (Fig. 1a). Interestingly, solid tumors seem to be more resistant to CvL, since no cytotoxicity was observed against adherent tumor cell lines from different histological origins (B16 murine melanoma, human renal carcinoma 786-O cell line and human prostate cancer cell line PC3) when incubated with CvL (10–150 μg ml^{-1}) for 24 h (Fig. 1, supplemental material). Taken together, these data point to a selective activity of CvL against K562 leukemia cells.

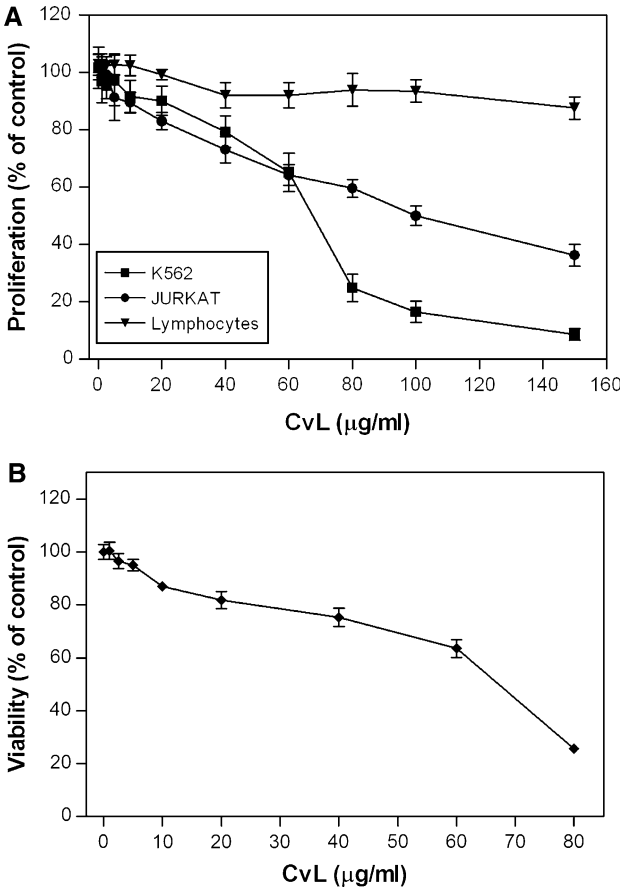

Fig. 1 Cytotoxicity of CvL in leukemia cells and normal peripheral blood lymphocytes. **a** K562 and JURKAT cells (2×10^4 ml^{-1}) were treated with different concentrations of CvL (1–150 μg ml^{-1}) for 72 h in 96-well microtiter plates. Peripheral blood mononuclear cells were collected from normal human donors and separated by density gradient as described in Sect. "Materials and methods". Leukocytes at a density of 1×10^6 plating ml^{-1} were cultured with 5 μg ml^{-1} of phytohemagglutinin in 96-well microtiter plates, in the presence and absence of different concentrations of CvL (1–150 μg ml^{-1}) for 72 h. Cell proliferation was assessed by the MTT reduction test. In the absence of compound, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction was considered as 100%. **b** K562 cells (2×10^4 ml^{-1}) were treated with different concentrations of CvL (1–80 μg ml^{-1}) for 72 h in 96-well microtiter plates. Cell viability was determined by the trypan blue exclusion method. In the absence of compound, the viability was considered as 100%. Results represent the mean ± standard deviation of three experiments run in four replicates ($P < 0.05$ compared with control cells)

Apoptosis induction by CvL in K562 cells

To understand the mechanism by which CvL caused viability loss in K562 cells, several experiments were carried out involving apoptosis. First, the occurrence of cell death was analyzed by flow cytometry after staining of cells with PI. Exposure to CvL significantly ($P < 0.001$) increased the percentage of cell death, represented by the sub-G_1 fluorescent peak, compared with control (Fig. 2a). The percentage of this sub-G_1 peak in the control cells was 14.1 ± 1.5% and shifted to 28.0 ± 0.6% in damaged cells (70 μg ml^{-1} of CvL). In order to verify if apoptosis could be a final cellular response of CvL activity, we monitored the nuclear changes by DAPI staining [26, 27]. As showed in Fig. 2d, control cells exhibited nuclei with dispersed chromatin (Fig. 2d, top). In contrast, cells treated with CvL for 72 h showed nuclei with different levels of chromatin condensation and nuclear fragmentation, as demonstrated by the fluorescent pattern of DAPI (Fig. 2d, bottom). To quantify apoptosis, K562 cells were incubated for 72 h in the presence of 50 and 70 μg ml^{-1} CvL, and the apoptotic indices were determined. CvL at 70 μg ml^{-1} significantly ($P < 0.001$) increased the number of apoptotic cells, comprising 43.0 ± 5.1% of the total cell population at 72 h (Fig. 2c). However, treatment with 50 μg ml^{-1} of CvL only slightly increased the amount of apoptosis observed in untreated

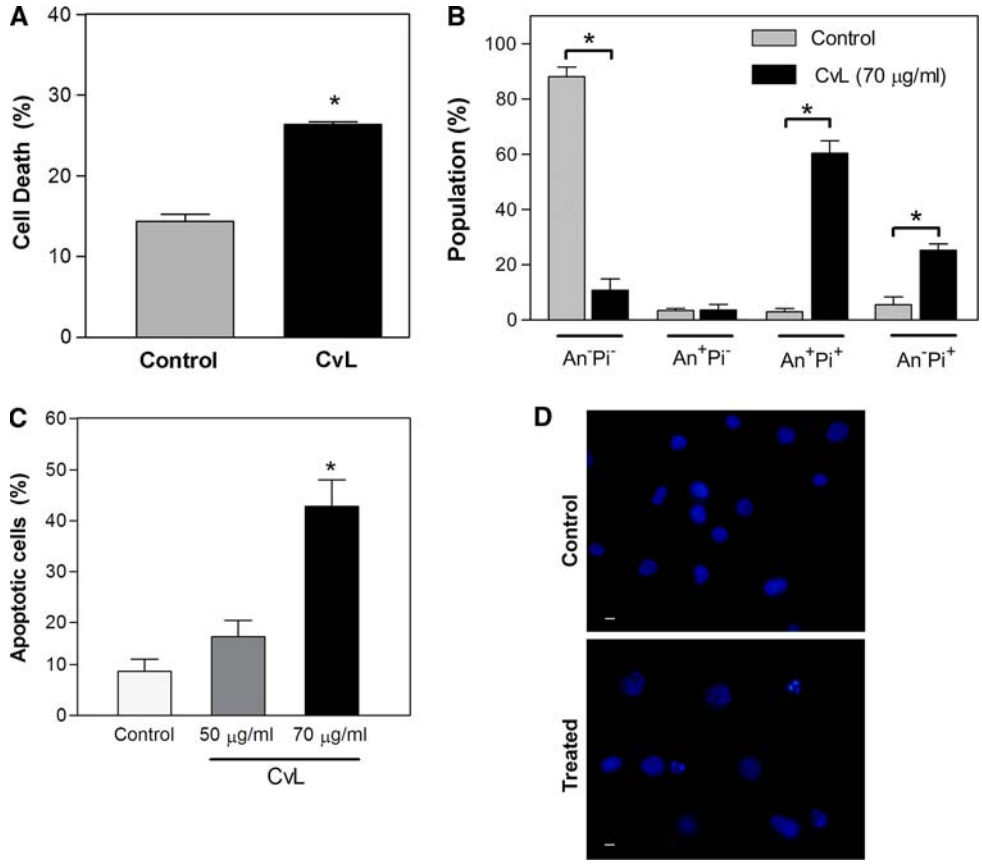

Fig. 2 Apoptosis induction by CvL in K562 cells. **a** Cell death in cultures of K562 cells treated with 70 μg ml^{-1} of CvL for 72 h. Cells were stained with propidium iodide and the percentage of cells in the sub-G_1 phase of cell cycle analyzed by flow cytometry. Data are representative of three independent experiments and express the mean ± standard deviation. **b** Cell samples were prepared as described in Sect. "Materials and methods", and Annexin V/PI-negative, Annexin V-positive, PI-positive, and Annexin V/PI-positive populations were analyzed by flow cytometry. Data are representative of three independent experiments and express the mean ± standard deviation. **c** Apoptosis indices of K562 cells exposed to 50 or 70 μg ml^{-1} of CvL for 72 h determined by DAPI staining. The apoptotic index was determined by counting 100 cells per field of view in five different fields per sample. Each sample was done in duplicate and the number of apoptotic cells was expressed as a percentage of the total number of cells. *Error bars* represent standard deviation. **d** Morphological features of CvL-induced apoptosis in K562 cells (2×10^4 ml^{-1}) treated with 70 μg ml^{-1} of CvL. (*top*) Micrograph of control cells stained with DAPI showing normal nucleus; (*bottom*) Cells treated with CvL for 72 h showing apoptotic nucleus. Representative photomicrographs of two independent experiments. *Bars* 20 μm. $^*P < 0.001$ compared with control

K562 control cells, presenting an apoptotic index of 15.5 ± 3.0% (Fig. 2c). Furthermore, analysis by flow cytometry of CvL-treated cells stained with annexin V-FITC/PI indicated that late apoptosis and necrosis were predominant as detected by the significant ($P < 0.001$) increase in annexin V-FITC/PI double positive and in PI positive populations, corresponding to 60.4 ± 4.5 and 25.3 ± 2.5% of cells, respectively (Fig. 2b).

Involvement of cathepsin B and TNFR1 in CvL-induced K562 cell death

The majority of apoptosis inducers can trigger cell death by caspase activation, so we next examined the effects of CvL on the activity of caspases after 72 h of treatment. First, to elucidate whether caspase family proteases are activated in the CvL-induced apoptotic process, their proteolytic activities were measured using colorimetric assays (Fig. 3a). Surprisingly CvL failed to induce caspase-8 (IETDase) and caspase-9 (LEHDase) activities. In addition, no significant effector caspase-3 (DEVDase) activity was detectable in K562 cells treated with CvL at both concentrations (50 and 70 μg ml^{-1}). The lack of caspase-3 activity was further demonstrated by flow cytometric analysis of the endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to aspartic acid 175 (Fig. 3b).

These results prompted us to investigate whether the apoptosis-inducing action of CvL could be due to proteases other than caspases. In particular, cathepsin B has been reported to contribute to non-classical induction of cell death [2, 29]. Thus, we examined this possibility using

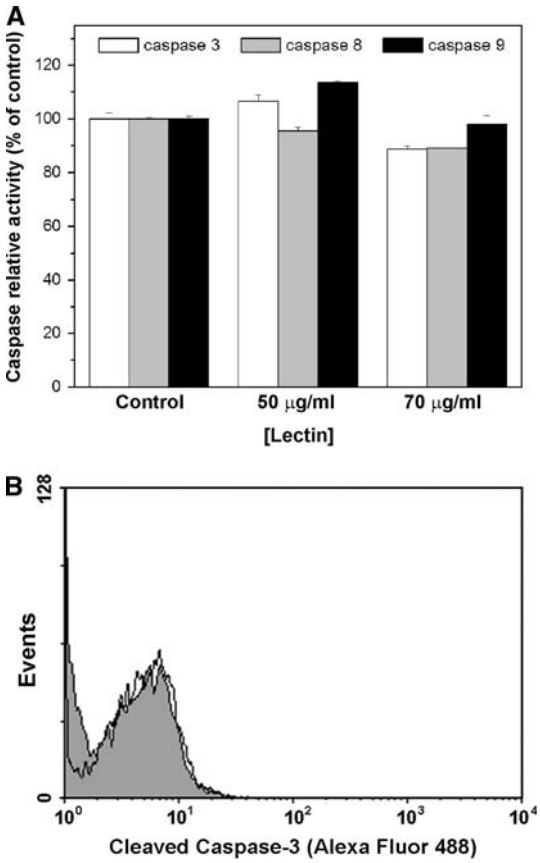

Fig. 3 CvL-induced K562 cell death is independent of caspases. **a** Caspase relative activity in K562 (2×10^4 ml^{-1}) cells after 72 h of treatment with 50 or 70 μg ml^{-1} of CvL for 72 h. Colorimetric assay was performed to determine caspase-3, -8 and -9 activities as described in Sect. "Materials and methods". The enzyme activities were calculated in pmol min^{-1} (mg protein)$^{-1}$ (extinction coefficient of *p*NA was 10,000 M^{-1} cm^{-1}) and expressed relative to controls. In the absence of CvL the activity was considered to be 100%. *Error bars* represent standard deviation. **b** Flow cytometric analysis of endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 in K562 cells. Cell samples were prepared as described in Sect. "Materials and methods", labeled with anti-cleaved caspase-3 (Asp175) Alexa Fluor 488-conjugated antibody and analyzed by flow cytometry. *Grey histogram* represents control cells treated with medium only. One representative histogram of three independent experiments for each sample is presented

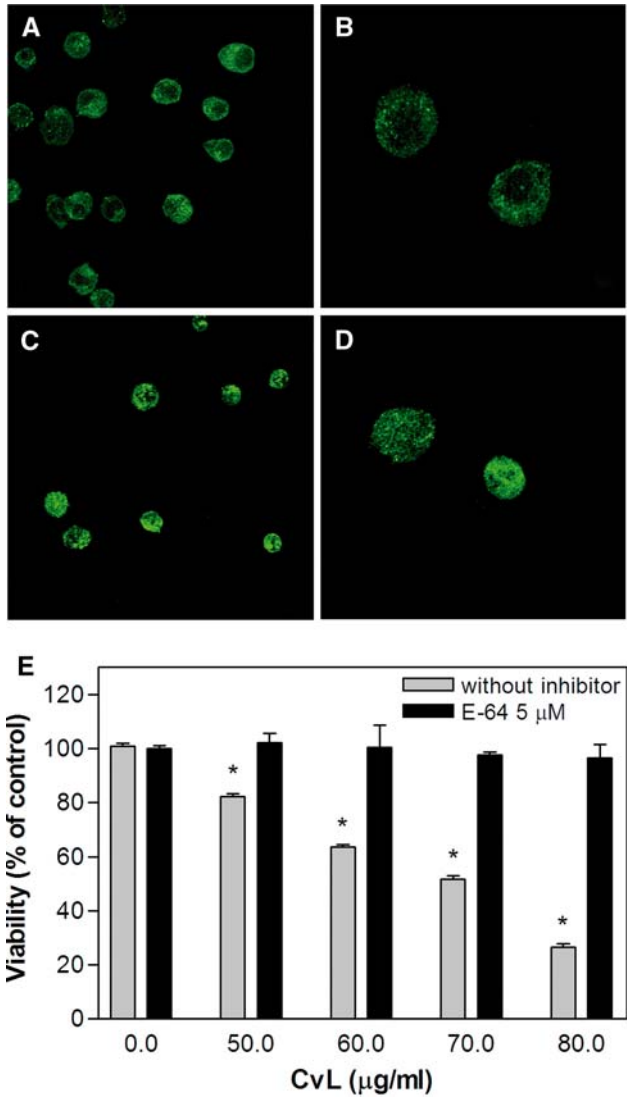

Fig. 4 Immunofluorescence staining of cathepsin B and microscopic analysis of K562 cells treated with CvL. (**a**, **b**) Control cells showing punctated cathepsin B fluorescence staining in the cytoplasm. (**c**, **d**) Cells (2×10^4 ml^{-1}) treated with 70 μg ml^{-1} of CvL for 72 h showing dispersed cathepsin B fluorescence. Cells treated with CvL or vehicle (control) were incubated with anti-cathepsin B antibody and labeled with anti-rabbit IgG Alexa Fluor 488-conjugated antibody. Cells were then analyzed using an inverted laser scanning confocal microscope with emission and excitation wavelengths of 515 and 491 nm, respectively. Representative photomicrographs of two independent experiments. **e** Effect of cathepsin inhibition on CvL-induced cell death in K562 cells. Cells (2×10^4 ml^{-1}) were pre-treated with 5 μM of the cathepsin inhibitor L-trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) for 2 h, and then incubated with different concentrations of CvL (50–80 μg ml^{-1}) for 72 h in 96-well microtiter plates. Cell viability was assessed by the MTT reduction test. In the absence of compound, the MTT reduction was considered as 100%. Results represent the means ± standard deviation of three experiments run in triplicate ($P < 0.05$ compared with control cells)

laser scanning confocal microscopy to visualize the cellular compartmentation of cathepsin B in K562 cells treated with the lectin. In control cells, the distribution of cathepsin B protein was more punctate and only localized in the cytosol (Fig. 4a, b). After treatment of K562 cells with 70 μg ml^{-1} CvL for 72 h, the fluorescence was distributed in the cytoplasm and also in the nuclear region, suggesting translocation of cathepsin B from the vesicular compartment into the cytoplasm during exposure to CvL (Fig. 4c, d).

In order to test if cysteine cathepsins could explain the cytotoxic effect of CvL, K562 cells were previously incubated with the broad-spectrum cathepsin inhibitor E-64 for 2 h. As shown in Fig. 4e, cell death was completely prevented by E-64, as measured by the MTT reduction endpoint. Moreover, this effect was evident even when high

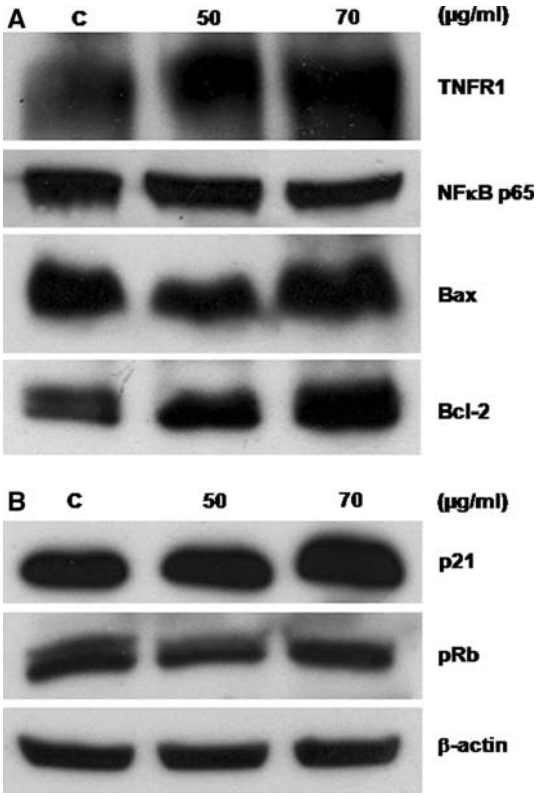

Fig. 5 Effects of CvL on the expression of proteins involved in the TNFR1 signaling, NFκB expression and cell cycle regulation in K562 cells. K562 cells (2×10^4 ml^{-1}) were treated for 72 h with 50 or 70 μg ml^{-1} of CvL. Equal amounts of total protein (50 μg) from cell lysates loaded per lane and blotted with anti-TNFR1, anti-NFκB p65, anti-Bcl-2 and anti-Bax (**b**); and anti-p21 and anti-pRb (**b**) antibodies. Equal loading was confirmed by showing equal β-actin levels. One representative immunoblot of three independent experiments is presented

concentrations of CvL were used in a 72-h treatment period. These observations indicate that cathepsin B could be, at least partially, implicated in CvL-mediated cell death.

Modulation of NFκB and TNFR1 expression by CvL in K562 cells

Recent reports highlight the role of cathepsin B in death receptor-induced apoptosis [2, 27, 29–31]. Thus, we checked the expression profile of TNFR1 in K562 cells treated with CvL. Western blotting was performed in K562 cells treated with 50 and 70 μg ml^{-1} of CvL. The analysis revealed that the expression of the TNFR1 increased in a dose-dependent manner after 72 h of treatment (Fig. 5a).

One of the events associated with members of the TNF receptor signaling is the activation of the anti-apoptotic transcriptional factor NFκB [32]. Studies of cell cultures and animal models revealed the activation of NFκB p65/RelA by BCR-ABL [33, 34]. In order to test if the increased TNFR1 level induced by CvL treatment affected NFκB cascade, the expression of p65 NFκB subunit was detected by Western blot analysis of protein samples from K562 cells. Treatment of K562 cells with 70 μg ml^{-1} of CvL resulted in a decreased expression of p65 subunit of NFκB, although the concentration of 50 μg ml^{-1} almost unaffected the subunit levels (Fig. 5a).

Expression levels of anti-apoptotic proteins such as Bcl-2 and Bcl-X_L were reported to be upregulated by the transcriptional factor NFκB [35, 36]. This class of pro-survival Bcl-2 members sequesters pro-apoptotic proteins of the Bax subfamily and ultimately prevents Bax or Bak activation/oligomerization, and consequently inhibits mitochondrial pro-apoptotic events, acting upstream of the effector caspase [31]. Although CvL treatment results in increased levels of Bcl-2, this effect was accompanied by enhanced expression of the pro-apoptotic protein Bax (Fig. 5a), implying that an alternative death receptor-mediated execution pathway or multiple signaling pathways is involved in CvL activity.

Finally, we investigated the effects of CvL on proteins involved in the cell cycle regulation (Fig. 5b). Western blot analysis showed a significant upregulation of p21 protein expression, indicating cell cycle arrest due to CvL treatment. In addition, a downregulation of pRb, which is a critical determinant in blocking DNA duplication, was detected. These findings were consistent with previous reports in the literature showing apoptosis induction and blockade of cell cycle progression at G_1 and/or G_2/M by other agglutinins such as Concanavalin A (Con A), rice bran agglutinin (RBA) and wheat germ agglutinin (WGA) in different cell systems [13, 37, 38].

Discussion

Extensive research in the last decades have unraveled the signal transduction pathways controlling cell death and the molecular core machinery responsible for cell demise, leading to numerous possibilities for pharmacological intervention and drug design. Among potential new drugs able to interfere with the deregulated growing of tumor cells, natural products have been regarded as important sources of novel chemotherapeutic agents and/or scaffolds for the development of compound libraries [39]. Lectins are a class of proteins widely spread in living organisms which have attracted attention for their promising application in cancer therapy, in part due to their action as potential activators of tumor cell death by triggering apoptotic signaling cascades [7, 12–15]. In the present study, a BCR-ABL expressing human erythroleukemia cell line (K562) was used as a model system of resistance to apoptosis induced by conventional chemotherapeutics [40], to investigate the molecular mechanisms involved in the antitumoral activity of CvL. The results

presented here show that CvL was cytotoxic to human leukemia (K562 and JURKAT) cells but not to mammalian solid tumor cell lines (B16 melanoma, 786-O renal carcinoma cells and PC3 prostate tumor cells). Major cytotoxicity of CvL was observed against erythroleukemia K562 cells, with an IC_{50} value of 70 μg ml^{-1}. In contrast, CvL was ineffective against normal human peripheral blood lymphocytes in the same range of concentrations tested. These results are particularly important, since most traditional chemotherapeutic drugs exhibit severe normal toxicity and cause undesirable side effects, thus limiting their application in clinical sets. Moreover, several relevant therapeutics in clinical practice or development do not lead to cures or long-term survival for most cancers and resistance may eventually develop. Therefore, it is clear that new agents with different mechanisms of action are desired, both for the treatment and as adjuvant to improve cancer therapy. A drastic decrease in K562 cell viability, together with a prominent increase in the sub-G_1 peak, was observed after 72 h of exposure to the lectin. In addition, cell death occurred with typical signs of apoptosis as reflected in increasing apoptotic index and annexin V-FITC/PI double-positive cells.

Of extreme relevance is that cell death induced by CvL is clearly different from that mediated primarily via caspases. The fact that CvL did not induce the activation of caspase-3, -8, and -9 raised the possibility that other non-caspase proteases are involved in CvL-induced K562 cell death. Although caspases are well established as the main players in apoptosis, other proteases such as calpains, cathepsins, and serine proteases may account for alternative types of programmed cell death [1]. Our findings that CvL triggers release of cathepsin B as evidenced by fluorescence microscopy, and that the irreversible cathepsin inhibitor E-64 completely blocks CvL-induced cell death lead us to suggest a central role for the lysosomal protease cathepsin B in mediating the cytotoxic effect of CvL. This is consistent with previous reports linking the action of lysosomal proteases, in particular cysteine cathepsins, with caspase-independent death programs [2, 3, 29, 30]. Further investigation of the possible events related to these effects revealed that CvL treatment induced an upregulation of TNFR1 and down-modulation of NFκB expression in K562 cells. In addition to the original view that death receptors trigger cell death by direct activation of the caspase cascade, several reports have implicated TNFR1 in death processes occurring in the complete absence of caspases. Furthermore, compelling evidence indicates that cathepsin B is a crucial downstream effector in this caspase-independent/receptor-induced cell death [1, 2, 29, 30]. Thus, cathepsin B can take over the role of the dominant execution protease in conservation of this alternative execution pathway. The data presented here suggest that activation of membrane death receptor may play a proximal role in lysosomal cathepsin B-mediated cell death induced by CvL in K562 erythroleukemia cells. Lysosomal permeabilization with subsequent release of cathepsin B in the cytosol has been implicated in activation of apoptotic cascades in other studies [27, 29, 30, 41], supporting the contention that lysosomal cell death pathway may be a therapeutic strategy to overcome caspase-deficient cell death [2].

Tumor cells have frequently impaired mechanisms of programmed cell death to benefit from a selective growth advantage. In addition, one of the events associated with members of the TNF receptor signaling is the activation of the transcriptional factor NFκB, which has been implicated in suppression of apoptosis, cell survival, proliferation, and resistance to chemotherapeutic agents [8]. Extensive studies in cell cultures and animal models reveal the critical role of p65/RelA NFκB in BCR-ABL-mediated tumorigenesis and transformation of haematopoietic cells [33, 34]. Also, expression levels of anti-apoptotic proteins such as Bcl-2 and Bcl-X_L were reported to be upregulated by the transcriptional factor NFκB [34, 35]. In parallel, the *BCR-ABL* oncogene prevents apoptotic death by conventional chemotherapeutics by inducing a *BCL2* expression pathway [36]. Recently, Dai et al. [42] reported that *BCL2* cooperates with *BCR-ABL* to promote leukemogenesis and a more aggressive tumor phenotype. Our observation of decreased expression of p65 NFκB subunit in lectin-treated cells could also contribute to the pro-apoptotic effect of CvL. Since increased levels of both proteins Bax and Bcl-2 were observed after treatment, it seems possible that CvL affects the balance between death inhibiting and death promoting signals in favor of increased death of K562 cells. These observations further support the idea that cathepsin B may play a major role in this process.

BCR-ABL is a select group of oncogenes that is capable of both inhibiting apoptosis and deregulating cell proliferation [43]. The combination of these activities is likely to be important for the progression of CML. Several lines of evidence support a role for NFκB activation by classical oncogenes such as *BCR-ABL* in cell cycle deregulation. Studies have demonstrated that NFκB is required to induce cyclin D1 expression and pRb hyperphosphorylation, and promote G_1 to S progression [44]. In the present study, Western blot analysis revealed that CvL caused increased expression of p21 and down-modulation of pRb, thus indicating cell cycle arrest. p21 is an inhibitor of kinase activities of cyclin/cyclin dependent kinase complexes, and it is known to regulate at both G_1/S and G_2/M cell cycle transitions [45]. Indeed, previous reports have shown that blockade of cell cycle progression contributes to the apoptotic process induced by lectins [13, 37, 38].

On the basis of these findings, we propose that CvL kills K562 cells via a caspase-independent mechanism, possibly in a still uncharacterized connection with the death receptor pathway. Moreover, these results point to a proximal role for cathepsin B in this death pathway.

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