Calpain Inhibitor II Induces Caspase-dependent Apoptosis in Human Acute Lymphoblastic Leukemia and Non-Hodgkin’s Lymphoma Cells as Well as Some Solid Tumor Cells

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INTRODUCTION

The identification and development of new potent drugs that can trigger apoptosis in ALL2 and NHL cells are focal points in translational leukemia/lymphoma research. We have recently discovered that calcium mobilizers can induce apoptosis in ALL and NHL cells in a PKC-dependent fashion (1, 2). Therefore, dual-function drugs such as calphestin C (1) or a combination of calcium mobilizers and PKC inhibitors (2) could be useful in the treatment of ALL.

In a continued effort to improve our understanding and knowledge of calcium-triggered apoptosis in ALL and NHL cells, we decided to investigate the role of calpain in this process. Calpain is a calcium-dependent cysteine protease (for reviews, see Refs. 3–6) that is implicated in calcium-dependent cell death (7–16). Calpain inhibitors are generally considered as inhibitors of calpain-mediated apoptosis (8, 10, 11, 15, 17, 18). In the present study, we treated ALL and NHL cells with ionomycin, an ionophore that induces calcium influx, in the presence and the absence of a peptidyl calpain inhibitor, CPI-2. Unexpectedly, we found that CPI-2 does not inhibit ionomycin-induced calcium-dependent apoptosis. Instead, CPI-2 triggered rapid apoptosis in ALL and NHL cells, including: ALL-1, a multidrug-resistant BCR-ABL fusion transcript-positive t(9;22) pro-B ALL cell line; RS4;11, a highly radiation-resistant MLL-AF4 fusion transcript-positive t(4;11) pre-pre B ALL cell line; RAMOS, a highly radiation-resistant and p53-deficient Burkitt’s lymphoma cell line; DAUDI, a Burkitt’s leukemia/lymphoma cell line; NALM-6, a pre-B ALL cell line; and JURKAT and MOLT-3, two T-lineage ALL/NHL cell lines. CPI-2-induced apoptosis in LYN-deficient and BTK-deficient subclones of the DT-40 lymphoma B cell line as effectively as it did in wild-type DT-40 cells. Thus, CPI-2-induced apoptosis is not dependent on the protein tyrosine kinases LYN or BTK.

Notably, caspase inhibitor I effectively inhibited CPI-2-induced apoptosis, suggesting that the inhibition of a CPI-2-susceptible protease results in caspase activation, leading to apoptosis in ALL/NHL cells. Unlike the high calpain-expressing ALL/NHL cell lines, myeloid leukemia cell lines HL-60/AML, K562/CML, and U937/AMML, or solid tumor cells, including breast cancer, prostate cancer, glioblastoma, and HeLa/epitheloid cancer, were not susceptible to the cytotoxicity of CPI-2. Taken together, our results identify calpain as a new molecular target for the treatment of ALL and NHL. CPI-2 and its analogues represent a promising new class of antileukemia/lymphoma agents that deserves further development.

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2 The abbreviations used are: ALL, acute lymphoblastic leukemia; PKC, protein kinase C; PTK, protein tyrosine kinase; BTK, Bruton’s tyrosine kinase; CPI-2, calpain inhibitor II; PI, propidium iodide; NHL, non-Hodgkin’s lymphoma; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; FBS, fetal bovine serum.

3 Unpublished data.
Fig. 1  Calpain expression in human cancer cells. Cells were stained with anti-calpain monoclonal antibody and FITC-labeled goat antimouse IgG and then imaged by confocal laser scanning microscopy, as described in “Materials and Methods.”
cell lines BT-20/breast cancer, PC-3/prostate cancer, U373/glioblastoma, and HeLa/epitheloid cancer were not susceptible to the cytotoxic activity of CPI-2. Taken together, our results identified calpain as a new molecular target for the treatment of ALL and NHL. CPI-2 and its analogues represent a promising new category of antileukemic agents that deserve further development.

MATERIALS AND METHODS

Cell Lines. The human ALL and NHL cell lines ALL-1, RS4;11, NALM-6, RAMOS, JURKAT, MOLT-3, and DAUDI, as well as the myeloid leukemia cell lines K-562, HL-60, and U-937, were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FBS (Summit Biotech, Ft. Collins, CO) and 100 units/ml penicillin + 100 μg/ml streptomycin (Life Technologies, Inc.). The wild-type LYN-deficient and BTK-deficient clones of the chicken lymphoma B cell line DT-40 (19) were cultured in the same culture medium supplemented with 1% chicken serum (Sigma Chemical Co., St. Louis, MO). The solid tumor cell lines U373 and HeLa were cultured in MEM supplemented with nonessential amino acids, Earl’s balanced salt solution, 1 mM sodium pyruvate, and 10% FBS. SQ-20B cells were grown in DMEM supplemented with 20% FBS (not heat-inactivated). PC-3 cells were cultured in Ham’s F12K medium supplemented with 10% FBS. All cell lines were maintained at 37°C in a humidified 5% CO2 atmosphere.

Inhibitors. CPI-2 (N-Ac-Leu-Leu-Met) and caspase inhibitor I [Z-Val-Ala-Asp(OMe)-CH2F] were purchased from Calbiochem (La Jolla, CA). Their stock solutions (10 mM in DMSO) were stored at -20°C.

Calpain Profiling of Cancer Cells. Immunofluorescence was used to examine the expression of calpain in leukemia and solid tumor cells, as described previously (2). Briefly, the solid tumor cells grown on coverslips and the leukemia cells adhered on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) were fixed in methanol at -20°C for 15 min. The cells were then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS in the presence of 0.1% sodium citrate for 15 min. The nonspecific binding sites were blocked with 2% BSA in PBS for 15 min. After a washing three times with PBS, the cells were interacted with 100 μl of anti-calpain monoclonal antibody (Chemicon International Inc., Temecula, CA; 1:100 dilution) at 37°C for 1 h and washed with PBS. FITC-conjugated goat antimouse IgG (Sigma Chemical Co.) at 2.5 μg/ml was interacted with the cells at 37°C for 40 min. Cells were then washed in PBS, and the coverslips were mounted with Vectashield containing PI (Vector Laboratories, Inc., Burlingame, CA). The fluorescence images of the cells were taken by a laser scanning confocal microscope (MRC 1024; Bio-Rad, Inc., Richmond, CA) and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Apoptosis Assays. Flow cytometric apoptosis assays were performed, as described previously (19). Briefly, loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation in apoptotic cells, thereby rendering MC540 binding an early marker of apoptosis (19). Plasma membrane permeability to PI (Sigma Chemical Co.) develops at a later stage of apoptosis (19). MC540 binding and PI permeability were simultaneously measured 24 h after exposure to CPI-2. Stock solutions of MC540 and PI, each at 1 mg/ml, were passed through a
0.22-μm filter and stored at 4°C in the dark. Shortly before analysis, cell suspensions (1 × 10⁶ cells/sample) were stained with 5 μg/ml MC540 and 10 μg/ml PI and kept in the dark at 4°C. Whole cells were analyzed with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). MC540 and PI emissions were split with a 600-nm short pass dichroic mirror; a 575-nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission, and a 635-nm band pass filter was used for PI emission.

DNA fragmentation in apoptotic cells was also documented using the in situ TUNEL assay and an in situ Cell Death Detection Kit (Boehringer Mannheim), as described (2). In brief, cells were centrifuged at 850 × g for 5 min after a 24-h treatment with CPI-2 at the indicated concentrations and then resuspended in PBS at a density of 5 × 10⁶ cells/ml. Samples (50 μl) of the cell suspensions were placed into a PAP Pen (Zymed Laboratories Inc., South San Francisco, CA)-circled area on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) that were coated for cell adhesion. The cells were allowed to adhere to the slide for 10 min, then washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. After washing the cells with PBS, the cells were permeabilized by incubation for 2 min with 100 μl of 1% Triton X-100 in PBS. The permeabilized cells were washed three times with PBS and treated for 1 h at 37°C with the reaction mixture containing terminal deoxynucleotidyl transferase and FITC-conjugated digoxigenin-11-UTP for labeling of the exposed 3’-hydroxyl ends of fragmented nuclear DNA. After washing the cells with PBS, a coverslip was mounted onto each slide with PI-containing mounting medium (Vector Laboratories, Inc.). The fluorescent images of the cells were acquired with a confocal laser scanning microscope (MRC 1024; Bio-Rad, Inc.). Because the apoptotic cells have many exposed 3’-hydroxyl ends that incorporate abundant amounts of FITC-labeled dUTP, they exhibit green fluorescence at a 488-nm excitation. In contrast, nonapoptotic cells incorporate minute amounts of FITC-labeled dUTP due to the lack of exposed 3’-hydroxyl ends and consequently have much less green fluorescence than apoptotic cells. All cells emit strong nuclear red fluorescence at a 514-nm excitation because of the binding of PI to nuclear DNA.

RESULTS AND DISCUSSION
Expression of Calpain in Human Cancer Cells. Human ALL and NHL cells were examined for calpain expression using immunofluorescent staining with a monoclonal anti-calpain antibody combined with confocal laser scanning micros-
copy. ALL and NHL cells expressed high levels of calpain (Fig. 1). Among the four solid tumor cell lines examined, only SQ-20B expressed high levels of calpain.

**CPI-2-induced Apoptosis in Human Cancer Cells.**

ALL (ALL-1, RS4;11, and JURKAT) and NHL (RAMOS and DAUDI) cells were treated by CPI-2 at 50 or 100 μM for 24 h and examined for apoptosis using a quantitative flow cytometric apoptosis detection assay, which simultaneously measures MC540 binding and PI permeability of the cells. As shown in Fig. 2, 85–99% of CPI-2-treated cells showed evidence of apoptosis, as measured by MC540 single fluorescence (i.e., right lower quadrant), for early apoptosis, or MC540/PI dual fluorescence (i.e., right upper quadrant), for advanced apoptosis. Notably, both RS4;11, a highly radiation-resistant MLL-AF4 fusion transcript-positive t(4;11) pre-pre B ALL cell line, and ALL-1, a multidrug-resistant BCR-ABL fusion transcript-positive t(9;22) pro-B ALL cell line, were exquisitely sensitive to CPI-2. RAMOS, a highly radiation-resistant and p53-deficient Burkitt’s lymphoma cell line (20), also showed high susceptibility to CPI-2. In contrast, myeloid leukemia cell lines K-562, HL-60, and U-937 showed little sensitivity to CPI-2 (Fig. 3). We have also examined the cytotoxicity of CPI-2 to solid tumor cells. Remarkably, SQ-20B cells, the multidrug- and radiation-resistant squamous cancer cells expressing high levels of calpain (Fig. 1), underwent apoptosis when treated by CPI-2 in a concentration-dependent fashion, whereas the other three cell lines expressing low levels of calpain were resistant to CPI-2 (Fig. 4).

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**Fig. 4** CPI-2 induces apoptosis in calpain-expressing SQ-20B cells. The solid tumor cell lines were treated with CPI-2 at various concentrations for 24 h and then examined for apoptosis by flow cytometry assay, as described in “Materials and Methods.” The percentage of apoptotic cells is the sum of the percentages of the cells at early and advanced apoptotic stages. The bars represent the mean values (±SEM) from three independent experiments.

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**Fig. 5** DNA cleavage in CPI-2-treated human ALL and NHL cells. Confocal fluorescent microscopic images of the cells labeled using the TUNEL assay after a 24-h treatment with 100 μM CPI-2, as described in “Materials and Methods.” The nuclei of all cells were stained by PI, which emits red fluorescence. The green nuclear fluorescence from the FITC-conjugated digoxigenin-11-UTP bound to 3’-hydroxyl ends of fragmented DNA indicates the apoptotic cleavage of nuclear DNA.
Apoptotic DNA fragmentation in CPI-2-treated cells was visualized by TUNEL assays combined with confocal microscopy (Figs. 5 and 6). In accordance with the flow cytometric evidence of apoptosis shown in Figs. 2 and 4, the strong yellow/green fluorescence in CPI-2-treated ALL/NHL and SQ20-B cells confirmed the apoptotic DNA cleavage in these cells.

**CPI-2-induced Apoptosis Does Not Require PTKs LYN or BTK.** PTKs LYN or BTK play important roles in the initiation of apoptotic signals in lymphoid cells (21–23). We, therefore, compared CPI-2 susceptibility of wild-type DT-40 lymphoma B cells to the CPI-2 susceptibility of LYN-deficient and BTK-deficient DT-40 clones. As evidenced in Figs. 7 and 8, the kinase-deficient clones of DT-40 cells were exquisitely sensitive to the cytotoxic activity of CPI-2. These results demonstrated that neither LYN nor BTK are required for CPI-2-induced apoptosis.

**A Caspase Mediates CPI-2-induced Apoptosis.** Caspases (also known as interleukin 1β-converting enzyme-like proteases) are a family of cysteine proteases that play pivotal roles in the induction of apoptotic signals in lymphoid cells (24–26; for reviews, see Refs. 27–29). By using a caspase inhibitor, we studied the involvement of caspases in CPI-2-induced apoptosis. As shown in Fig. 9, caspase inhibitor I (z-VAD-FMK), a competitive and irreversible broad spectrum inhibitor of caspases (Ref. 30; at a 50-μM concentration) inhibited CPI-2-induced apoptosis. This observation provides evidence that an apoptosis-promoting caspase system is activated after calpain inhibition with CPI-2. This previously unknown cross-talk between calpain and caspases seems to be important for regulation of apoptosis in neoplastic lymphoid cells.

In summary, our results show that human ALL and NHL cells express high levels of the cysteine protease calpain and calpain plays an important role for their survival. Inhibition of calpain with CPI-2 triggers apoptosis in these cells in a PTK-independent fashion. Therefore, calpain represents a new molecular target for the treatment of ALL and NHL. Further
development of CPI-2 and its analogues may lead to the design of effective salvage treatment programs for patients with recurrent or therapy-refractory ALL and NHL. This study extends our previous work aimed at identification of new agents that can trigger apoptosis in ALL and NHL cells.

REFERENCES


Fig. 8 DNA cleavage in CPI-2-treated DT-40 chicken lymphoma cells. Confocal fluorescent microscopic images of DT-40 cells labeled with the TUNEL assay after a 24-h treatment with 50 μM CPI-2, as described in “Materials and Methods.” The nuclei of all cells were stained by PI, which emits red fluorescence. The green nuclear fluorescence from the FITC-conjugated digoxigenin-11-UTP bound to 3'-hydroxyl ends of fragmented DNA indicates the apoptotic cleavage of nuclear DNA.


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