Z-Phe-Gly-NHO-Bz, an Inhibitor of Cysteine Cathepsins, Induces Apoptosis in Human Cancer Cells

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ABSTRACT

An increasing number of studies indicate that cysteine cathepsins contribute to cancer progression, invasion, and metastasis. Here we provide experimental evidence that the cathepsin inhibitor Z-Phe-Gly-NHO-Bz induces rapid apoptotic death in human cancer cell lines. Notably, the Z-Phe-Gly-NHO-Bz-induced apoptosis exhibited independence of p53, caspases, and mitogen-activated protein (MAP) kinases. Taken together, our results prompt the hypothesis that cysteine cathepsin(s) is a universal survival factor for cancer cells, and its inhibition leads to cancer cell apoptosis. The exquisite sensitivity of human cancer cells to CATI-1 indicates that this compound and its derivatives may provide the basis for new treatment programs against a broad spectrum of malignancies.

INTRODUCTION

Cathepsins belong to the papain superfamily of lysosomal cysteine proteases. Among the various cathepsins, cathepsin B is the most extensively investigated cysteine protease. Cathepsin B is abundantly expressed in cancer cells including breast and prostate cancer cells, glioblastoma cells, head and neck cancer cells (reviewed in Refs. 1–4), and cervical cancer cells (5) and has been implicated in degradation of the interstitial matrix and basement membranes, allowing cancer cells to invade locally and metastasize to distant sites (6–12). Cathepsin expression in cancer cells is associated with poor treatment outcome of patients with breast cancer, lung cancer, brain tumor, and head/neck cancer (1). Recently, forced expression of cathepsin B has been shown to rescue cells from serum deprivation-induced apoptotic death (13), and antisense oligonucleotides of cathepsin B induced apoptosis (14). These recent findings indicate that cathepsins have an antia apoptotic function that is in apparent contradiction with earlier reports that suggested that cathepsin B is a mediator of apoptosis (15, 16).

MATERIALS AND METHODS

Cell Cultures. SQ20B 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. NCI-H520 cells were cultured in RPMI 1640 supplemented with 10% FBS. BT-20, DU145, U373, and HeLa cells were cultured in MEM supplemented with non-essential amino acids, Earl’s BSS, 1 mM sodium pyruvate, and 10% FBS. All of the cell lines were cultured at 37°C in a humidified 5% CO2 atmosphere.

Materials. CATI-1 and CAS-1, as well as the MAP kinase inhibitors SB 202190 and PD 98059, were purchased from Calbiochem (La Jolla, CA). MC540 and PI were purchased from Sigma Chemical Co. (St. Louis, MO).

Clonogenic Assays. After treatment with CATI-1, cancer cells were resuspended in clonogenic medium consisting of cell culture medium and 0.9% methylcellulose. Cells were plated in duplicate 35-mm Petri dishes at 40,000 cells/dish and cultured in an incubator for 5–7 days. Colonies were enumerated using an inverted phase microscope. Results were expressed as a percentage of inhibition of colony-forming cancer cells.

Apoptosis Assays. Loose packing of membrane phospholipid head groups and cell shrinkage precede DNA frag-
mentation apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (18). Plasma membrane permeability to PI develops at a later stage of apoptosis (18). MC540 binding and PI permeability of the cells were simultaneously measured by flow cytometry 24 h after exposure to the apoptotic reagents. 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 containing 1 × 10^6 cells 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) using the 488-nm excitation from an argon laser. 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 photonmultiplier tube to measure MC540 emission, and a 635-nm band pass filter was used for PI emission. For each experiment, 10,000 cells were analyzed by FACS, and the percentage of cells at early (MC540 fluorescence only) and advanced (dual fluorescence of MC540 and PI) stages of apoptosis were obtained.

DNA cleavage in apoptotic cells was assayed by the in situ TdT-mediated dUTP nick end labeling using an in situ Cell Death Detection kit (Boehringer Mannheim) as described (19). In brief, cancer cells were detached by trypsin-EDTA, centrifuged at 850 × g for 5 min, and then resuspended in PBS at a density of 5 × 10^6 cells/ml. Fifty 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 was coated for cell adhesion. The cells were allowed to adhere to the slide for 10 min and then washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. After washing two times with PBS, the cells were permeabilized with 100 μl of 20 mM SDS in PBS for 10 min. The permeabilized cells were washed three times with PBS and incubated for 1 h at 37°C with the reaction mixture containing TdT 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 the slides with PI-containing mounting medium (Vector Labs, Burlingame, CA). The fluorescent images of the cells were acquired with a confocal laser scanning microscope (MRC 1024; Bio-Rad, Inc., Richmond, CA). Because the apoptotic cells have fragmented DNA with exposed 3’-hydroxyl ends incorporating abundant amounts of FITC-labeled dUTP, they exhibit green fluorescence. In contrast, nonapoptotic cells incorporate only insignificant amounts of FITC-labeled dUTP because of the lack of exposed 3’-hydroxyl ends in intact DNA and consequently have much less green fluorescence than apoptotic cells. On the other hand, the DNA-bound PI emits strong red nuclear fluorescence from all cells.

**Table 1** Inhibition of cancer cell colony formation by CATI-1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean no. of colonies</th>
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</tr>
</thead>
<tbody>
<tr>
<td>U373 (brain tumor)</td>
<td>0 (1664, 1675, 1652)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 (76, 84, 67)</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>BT-20 (breast cancer)</td>
<td>0 (3656, 3480, 3744)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50 (177, 154, 200)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SQ20B (neck cancer)</td>
<td>0 (3072, 2976, 3168)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50 (1838, 1732, 1944)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

**Fig. 1** Concentration dependence of CATI-1-induced apoptosis in human tumor cells. The cells were treated with CATI-1 at the indicated concentrations for 24 h and assayed for apoptosis using FACS. The percentage of apoptosis represents the total percentage of apoptotic cells at early and advanced stages of apoptosis. Data represent the means from three to five independent experiments; bars, SE.
RESULTS AND DISCUSSION

Cytotoxic Activity of Cathepsin Inhibitor CATI-1 against Cancer Cells. CATI-1 is a specific and irreversible inhibitor of cysteine cathepsins (17). We first investigated the effects of CATI-1 on tumor cell growth using in vitro clonogenic assays. The tumor cell lines used were U373 (glioblastoma), BT-20 (breast cancer), and SQ20B (squamous cell carcinoma). The cells were treated with the indicated concentrations of CATI-1 for 24 h and then cultured in clonogenic medium for 5–7 days. As shown in Table 1, treatment with CATI-1 inhibited the clonogenic growth of BT-20 and U373 as well as SQ20B cells in a concentration-dependent fashion. Ninety to 100% inhibition was achieved at a 50 μM concentration of CATI-1. These results uniquely indicate that the clonogenic cells are not spared from the cytotoxic activity of CATI-1.

CATI-1 Induces Apoptosis in Human Tumor Cells. We next examined the activity of CATI-1 against six different human cancer cell lines, including BT-20 (breast cancer), PC-3 (prostate cancer), U373 (glioblastoma), SQ20B (squamous cell carcinoma), HeLa (cervix cancer), and DU145 (prostate cancer), using a quantitative flow cytometric apoptosis detection assay. As shown in Fig. 1, exposure to CATI-1 induced apoptosis in all cancer cell lines tested in a concentration-dependent fashion, with EC_{50}s ranging from 22 to 47 μM. Representative FACS-correlated two-color displays are shown in Fig. 2. In contrast, normal peripheral blood mononuclear cells did not undergo apoptosis after CATI-1 treatment (data not shown).

The ability of CATI-1 to induce apoptosis in cancer cells was further confirmed using TdT-mediated dUTP nick end labeling assays combined with confocal microscopy. In accordance with the flow cytometric evidence of apoptosis shown in Fig. 2, the strong yellow/green fluorescence from nuclei of CATI-1-treated cancer cells confirmed the apoptotic DNA cleavage in these cells (Fig. 3). Furthermore, in contrast to the smooth and round nuclei of vehicle-treated control cells, the nuclei of CATI-1-treated cells were smaller and had an irregular shape, consistent with apoptotic shrinkage.

p53 Is Not Required for CATI-1-induced Apoptosis. Several chemotherapeutic drugs induce apoptosis in human cancer cells in a p53-dependent fashion, and loss of p53 function has been associated with drug resistance (20–24). To investigate whether p53 is required for CATI-1-induced apoptosis, we first examined the effects of CATI-1 on the NCI-H520 cell line, a lung squamous carcinoma cell line with markedly reduced p53 expression levels. We found that CATI-1-treated NCI-H520 cells underwent apoptosis with an EC_{50} of 55 μM (Fig. 4A). Furthermore, CATI-1-treated p53-deficient chronic myeloid leukemia cell line K562 also underwent apoptosis (Fig. 4B). Thus, p53 does not appear to be essential for CATI-1-induced apoptosis in human cancer cells.

CATI-1-induced Apoptosis Is Not Mediated by Caspases. Caspases are members of a family of interleukin-1β-converting enzyme-like cysteine proteases and play a pivotal role in apoptosis (Refs. 25–30; reviewed in Refs. 31–33). We next set out to study whether caspases are mediators for CATI-1-induced apoptosis by using CAS-1, a specific and irreversible broad spectrum inhibitor of caspases (24, 34–37). To this end, cancer cells were treated by 50 μM CATI-1 in the absence and presence of CAS-1 at concentrations ranging from 10 to 50 μM. Surprisingly, the caspase inhibitor CAS-1 had no detectable impact on CATI-1-induced apoptosis in any of the cell lines studied (Fig. 5). The fact that CAS-1 did not inhibit CATI-1-induced apoptosis indicates that CATI-1-induced apoptosis is not mediated by caspases.

MAP Kinases Do Not Mediate or Regulate CATI-1-induced Apoptosis. MAP kinases are key regulators of apoptotic signals (38, 39). The effects of MAP kinases on CATI-1-triggered apoptosis have been evaluated in the current work.
by using MAP kinase inhibitors PD 98059, a selective inhibitor of MAP kinase kinase (MEK), and SB 202190, a potent inhibitor of p38 MAP kinase. These two inhibitors neither enhanced nor attenuated the magnitude of apoptosis induced by CATI-1 in SQ20B cells (Fig. 6). Therefore, MAP kinases are not required for the apoptotic process triggered by CATI-1.

The identification and development of potent new anticancer drugs are focal points in translational cancer research. An increasing number of studies have implicated cysteine cathepsins in cancer progression, invasion, and metastasis. In this report, we have presented experimental evidence that the cysteine cathepsin inhibitor CATI-1 triggers apoptosis in human cancer cells. These results prompt the hypothesis that cysteine cathepsins have antiapoptotic survival-promoting functions in human cancer cells. However, our results do not exclude the possibility that the molecular mechanism of the CATI-1 cyto-

Fig. 3  DNA cleavage in CATI-1-treated cells. The fragmented DNA was labeled by FITC-conjugated digoxigenin-11-UTP in the presence of TdT, and the fluorescent images of cell nuclei were taken by laser scanning confocal microscopy, as described in “Materials and Methods.” Of the images, the red fluorescence from PI marks nuclei of all cells, and the yellow/green fluorescence from FITC-conjugated digoxigenin-11-UTP coupled to the 3'-hydroxyl end of DNA fragments indicates apoptotic fragmentation of DNA.

Fig. 4  p53 is not required for CATI-1-induced apoptosis. Cancer cells of NCI-H520 (A), a lung squamous carcinoma cell line expressing a remarkably reduced amount of p53 mRNA, and K562 (B), a p53-null human chronic myeloid leukemia cell line, were treated with CATI-1 at the indicated concentrations for 24 h and analyzed for apoptosis using FACS. The percentage of apoptosis represents the total percentage of apoptotic cells at early and advanced stages of apoptosis. Data represent means from three independent experiments; bars, SE.
toxicity is unrelated to the inhibition of cathepsins. Additional studies will be required to decipher the exact mode of action of this promising anticancer agent.

REFERENCES
Fig. 6 MAP kinases have no effect on CAT1-1-induced apoptosis. SQ20B cells (A) and HeLa cells (B) were treated with CAT1-1 at the indicated concentrations for 24 h in the absence and the presence of 20 μM PD 98059 or SB 202190. Apoptosis was analyzed by FACS. The percentage of apoptotic cells at early and advanced stages of apoptosis. Data represent the means from three independent experiments; bars, SE.
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