

Caffeic Acid Phenethyl Ester Induces Apoptosis of Human Pancreatic Cancer Cells Involving Caspase and Mitochondrial Dysfunction

Ming-Jen Chen^a Wen-Hsiung Chang^a Ching-Chung Lin^a Chia-Yuan Liu^a
Tsang-En Wang^a Cheng-Hsin Chu^a Shou-Chuan Shih^a Yu-Jen Chen^b

^aDivision of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital and Mackay Medicine, Nursing and Management College, Taipei, and ^bDepartment of Radiation Oncology, Mackay Memorial Hospital, Taipei, Taiwan (ROC)

Key Words

Pancreatic cancer · Caffeic acid phenethyl ester · Apoptosis · BxPC-3 cells · Caspase · Mitochondrial dysfunction

Abstract

Aims: This study aimed to investigate the effect of caffeic acid phenethyl ester (CAPE), an active component isolated from honeybee propolis, in inducing apoptosis in human pancreatic cancer cells. **Methods:** Inhibition of viability of BxPC-3 and PANC-1 cell lines induced by CAPE was estimated by a trypan blue dye exclusion test. The type of cell death in BxPC-3 after CAPE treatment was characterized by observation of morphology, sub-G₁ DNA content, annexin-V/PI staining, caspase-3 and caspase-7 assay, and DNA agarose gel electrophoresis. **Results:** CAPE (10 μg/ml) resulted in marked inhibition of viability of BxPC-3 (80.4 ± 4.1%) and PANC-1 (74.3 ± 2.9%) cells. CAPE induced a time-dependent increase in hypodiploid percentage and a significant decrease in mitochondrial transmembrane potential in BxPC-3 cells. It induced morphological changes of typical apoptosis, but no DNA fragmentation was noted by DNA electrophoresis. The inhibition of growth and increased in the proportion of sub-G₁ cells was partially blocked by pretreatment with the pan-caspase inhibitor Z-VAD-fmk (50 μM) in BxPC-3 cells

indicating a caspase-related mechanism in CAPE-induced apoptosis. Caspase-3/caspase-7 activity was approximately 2 times greater in CAPE-treated BxPC-3 cells compared with control cells. **Conclusions:** These results suggest that CAPE is a potent apoptosis-inducing agent. Its action is accompanied by mitochondrial dysfunction and activation of caspase-3/caspase-7.

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Introduction

Pancreatic cancer remains a major unsolved health problem. Late diagnosis, rapid progression and resistance to chemo- and radiotherapy are all responsible for the high mortality of pancreatic cancer [1], which has a median survival of less than 6 months and a 5-year survival of less than 4% [2]. Surgery remains a curative option for early disease, [3] but fewer than 15% of patients have resectable tumors. Almost all patients with pancreatic cancer develop metastases. The effects of treatment remain very limited, so that early detection at a resectable stage [4] or a novel agent for this devastating disease is urgently needed [5]. Although gemcitabine is currently the drug of choice for chemotherapy [6], its <20% objective re-

response rate is dismal [7–9]. It is therefore worth investigating in the pursuit of treatment for pancreatic cancer.

Advances in molecular biology have improved our understanding of the pathogenesis of pancreatic cancer [10], allowing identification of potential molecular targets for new types of therapy. Various genetic disturbances have been found, including K-ras mutation [11, 12], overexpression of growth factor receptors, activation of downstream cancer-associated pathways (PI3K/Akt, MAP kinases, NF- κ B) [13–15] and defective apoptosis [16]. Thus far, attempts to develop new treatments, either in studies of cells or animal models, have targeted growth factor receptor inhibitors [17, 18] or involved gene therapy, antisense therapy, immunotherapy, inhibition of matrix metalloproteinase and cyclo-oxygenase-2 [19], and induction of apoptosis. Resistance to apoptosis is a major cause of the insensitivity of pancreatic cancers to conventional therapies. Hence, one strategy is to search for agents that can overcome this resistance.

Propolis is a wax-like resinous substance collected by honeybees from tree buds or other botanical sources and used as cement and to seal cracks or open spaces in the hive. It has been a popular folk medicine through the ages, and claimed the beneficial effect on human health. Caffeic acid phenethyl ester (CAPE), an extract of honeybee propolis with well-known antioxidant activity [20], can be synthesized by simple esterification of caffeic acid. It is effective in protecting against ischemia-reperfusion injury by reduction of oxidative stress and neutrophil accumulation [21, 22]. It inhibits certain enzyme activities such as xanthine oxidase [23] and cyclo-oxygenase [24]. It has been reported to have anti-inflammatory properties involving the inhibition of transcription factor NF- κ B activation [25]. As an antitumor agent, CAPE suppresses intestinal carcinogenesis on animals bearing a germline mutation in the *apc* gene [26]. Our previous work showed that CAPE very quickly entered HL-60 cells, then caused glutathione depletion [27], mitochondrial dysfunction, caspase-3 activation, and subsequent apoptosis [28]. CAPE is also known to inhibit NF- κ B and induce apoptosis via Fas signal activation in human breast cancer MCF-7 cells [29]. In vivo, intraperitoneal injection of CAPE (10 mg/kg/day) to BALB/c mice reduced the pulmonary metastatic capacity of CT26 cells in association with a decreased plasma VEGF level [30].

In this study, we aimed to investigate the ability of CAPE to induce apoptosis in human pancreatic cancer cells.

Materials and Methods

Cell Lines and Culture Conditions

BxPC-3 cells, derived from a moderately differentiated human pancreatic adenocarcinoma, and PANC-1 cells, from a poorly differentiated human pancreatic epithelioid carcinoma, were purchased from the American Type Culture Collection (ATCC, Rockville, Md., USA). BxPC-3 cells were cultured in RPMI1640 medium (Biosource, Camarillo, Calif., USA) and PANC-1 cells in DMEM (Biosource) supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Israel) at 37°C in a humidified 5% CO₂ incubator. The cells were passaged every 2–3 days with TEG solution (0.25% trypsin, 0.1% EDTA, and 0.05% glucose in Hanks' balanced salt solution) and maintained in exponential growth.

CAPE Treatment

CAPE (purity \geq 99%) was purchased from Sigma Chemical Co. (St Louis, Mo., USA) and was dissolved in DMSO or absolute ethanol (stock solution). For induction of apoptosis, the pancreatic cancer cells were cultured in a 96-well microplate for 18 h at an initial concentration of 5×10^5 /ml and grown at 37°C in a humidified 5% CO₂ incubator. Various concentrations of CAPE (2.5, 5, 10 and 20 μ g/ml) with a final ethanol concentration of 0.1% v/v were added to the wells. We previously found that this concentration of ethanol had no significant effect on the growth of cells [28]. Gemcitabine (1 μ M) (2',2'-difluorodeoxycytidine, Gemzar, Eli-Lilly, Indianapolis, Ind., USA) was used as a positive control. In another experiment, the pan-caspase inhibitor Z-VAD-fmk (50 μ M) (R&D Systems, Minneapolis, Minn., USA) was added to the cells 2 h before CAPE treatment to examine the role of caspases in CAPE-induced growth inhibition.

Assessment of Inhibition of Cell Viability

After treatment with the various concentrations of CAPE or gemcitabine 1 μ M, cells were harvested at various times (from 24 h to 3 days). The numbers of viable cells were counted using a trypan blue dye exclusion test. Trypan blue exclusion test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Viable cell number was counted. We also used MTT assay to confirm the data. Cells with the same treatment were resuspended in fresh medium and cultured for 2 days before the assay. And cells were incubated with the MTT (tetrazolium compounds) for 4 h, lysed, and the dark crystals were solubilized with an ELISA reader at a wavelength of 570 nm. After treatment with CAPE for 48 h, a sample of cells were collected and stained with Liu's and Hoechst 33342 stain and examined under an Olympus light microscope at a magnification of \times 1,000.

Cell Cycle Analysis

The proportion of cells with hypodiploid DNA was measured using propidium iodide (PI) staining and flow cytometry. BxPC-3 cells were harvested, washed with PBS, and re-suspended (10^5 /ml) in 1.5 ml hypotonic fluorochromic solution (50 μ g/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) (Sigma, St. Louis, Mo., USA) for 1 h at 4°C in the dark. The PI fluorescence of individual nuclei was analyzed by a FACScaliber flow cytometer (Becton Dickinson, Lincoln Park, N.J., USA). As an estimate of the proportion of apoptotic cells, the percentage of hypodiploid cells

in a population of 10,000 cells was calculated using ModFIT cell cycle analysis software Version 2.01.2 (Becton Dickinson).

Quantitation of Apoptosis by Hoechst 33342 and Annexin-V-FITC/PI Double Staining

To examine cells for evidence of apoptosis, they were simultaneously stained with Annexin V-fluorescent isothiocyanate (FITC) conjugate and with PI. Viable cells take up neither dye (FITC-/PI-), cells undergoing early apoptosis fluoresce green (FITC+/PI-), and cells in the late apoptotic phase take up both FITC and PI and therefore demonstrate both green and red fluorescence (FITC+/PI+). Untreated cells, BxPC-3 cells treated with CAPE at two concentrations (10 and 20 $\mu\text{g/ml}$), and cells treated with gemcitabine (1 μM) were incubated for 24 h. After harvesting and washing with PBS, cells were resuspended in 100 μl Annexin V binding buffer (containing 10 mM HEPES/NaOH, pH 7.4, 1.5 M NaCl, and 10 mM CaCl_2) and subsequently incubated with 5 μl of FITC-conjugated Annexin V (TACS Annexin V-FITC Apoptosis Detection Kit; R&D Systems, Minneapolis, Minn., USA) for 15 min at room temperature. After Annexin-V-FITC staining, 400 μl of Annexin V binding buffer containing PI were added. Within 1 h, the cells were then analyzed by flow cytometry. As an estimate of the proportion of apoptotic cells after Hoechst 33342 staining, cells treated with CAPE exhibited condensed and fragmented nuclei under immunofluorescent microscopic observation were counted as apoptotic cells and calculated their percentage.

DNA Extraction and Gel Electrophoresis

BxPC-3 cells ($10^5/\text{ml}$) were lysed with 0.5 ml lysis buffer containing 5 mM Tris-borate at pH 8.0, 0.25 ml Nonidet P-40, and 1 mM EDTA, followed by the addition of RNase (Sigma) to a final concentration of 20 $\mu\text{g/ml}$, and incubated at 37°C for 1 h. Cells were further treated with proteinase K (300 $\mu\text{g/ml}$) for another 1 h, and the DNA was then isolated. Electrophoresis was carried out on 1.5% agarose gel in 5mM Tris-borate buffer (pH 8.0) containing 1 μM EDTA. The DNA on the gel was stained with ethidium bromide and visualized by UV illumination.

Transmembrane Mitochondrial Potential

The mitochondrial transmembrane potential was assessed by using a FACS caliber flow cytometer (Becton Dickinson). Briefly, PBS-washed BxPC-3 cells were incubated with 40 nM 3, 3'-di-hexyloxycarbocyanine iodine (Molecular Probes, Eugene, Oreg., USA) for 15 min at 37°C in the dark. The intensity of the green fluorescence (FL-1 channel) was measured to indicate changes in mitochondrial transmembrane potential with excitation at 488 and emission at 530 nm.

Western Blotting

Whole-cell lysates were prepared from BxPC-3 cells after treatment with 10 $\mu\text{g/ml}$ CAPE for 0, 2, 16, 24 and 48 h. The following reagents were added for 30 min on ice to extract total protein: 200 μl of lysis buffer containing 50 mM Tris pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 1% Nonidet P-40, and 10 $\mu\text{g/ml}$ leupeptin. The cell lysates were centrifuged at 12,000 rpm for 15 min and the protein concentration was determined with a bicinchoninic acid assay kit (Pierce, Rockford, Ill., USA). Equal amounts of protein (50 μg in each lane) were electrophoresed in 10% SDS-polyacrylamide gel at a constant voltage of

100 V and transferred onto a blotting membrane. The membrane was blocked with 5% de-fatted milk and then immunoblotted with primary antibodies including anti-Bcl-2 (MBL, Japan), anti-Bax (Upstate, Charlottesville, Va., USA), Smac/Diablo mouse monoclonal antibody (Cell Signaling, Danvers, Mass., USA), anti-HtrA2/Omi (MBL, Japan), rabbit anti-AIF polyclonal antibody (BD Biosciences, Franklin Lakes, N.J., USA), mouse anti-cytochrome C monoclonal antibody (BD Biosciences, Franklin Lakes, N.J., USA) and anti-poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling) at room temperature for 2 h. This was followed by addition of horseradish peroxidase-labeled second antibodies (Chemicon, Single Oak Drive, Temecula, Calif., USA) and developed using the enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, N.J., USA). Equal protein sample loading was monitored by hybridizing the same membrane filter with β -actin antibody as an internal control.

Caspase Substrate Activity Assay

BxPC-3 cells were harvested, washed, and counted. The cells were resuspended with 50 ml of chilled cell lysis buffer and incubated on ice for 10 min. 50 μl of 2 \times reaction buffer, 1 μl DTT and 5 μl 1mM AFC conjugated substrates were added to each sample. The samples were incubated for 1–2 h at 37°C in the dark. After incubation, the cells were harvested and the pellets assayed for caspase activity using a caspase fluorometric substrate set II plus (Medical & Biological Laboratories, Japan). The samples were read in a fluorometer with a 400-nm excitation filter and 505-nm emission filter. The degree of increased caspase activity was determined by comparison with an untreated control.

Statistics

Data from three or more separate experiments were expressed as mean \pm standard error. Student's t test was used to analyze the differences in growth inhibition and apoptosis between CAPE-treated BxPC-3 cells with and without Z-VAD-fmk pretreatment.

Results

Effect of CAPE Treatment on Inhibition of Viability of BxPC-3 and PANC-1 Cells

By using trypan blue exclusion test, the viability of BxPC-3 and PANC-1 cells was significantly inhibited by CAPE in a dose- and time-dependent manner (fig. 1a, b). A concentration of 10 $\mu\text{g/ml}$ of CAPE caused marked inhibition of viability in both BxPC-3 ($80.4 \pm 4.1\%$) and on PANC-1 ($74.3 \pm 2.9\%$) cells at 72 h. We also performed MTT assay to verify the reduction of cell viability by CAPE treatment in BxPC-3 and PANC-1 cells. These data are shown in figure 1c, d. The concentration required to inhibit viability by 50% was 4.5 $\mu\text{g/ml}$ for BxPC-3 and 6.0 $\mu\text{g/ml}$ for PANC-1 cells. In this study, 10 $\mu\text{g/ml}$ of CAPE was more effective than 1 μM of gemcitabine in inhibiting the viability of PANC-1 cells (74.3 ± 2.9 vs. $49.3 \pm 3.8\%$).

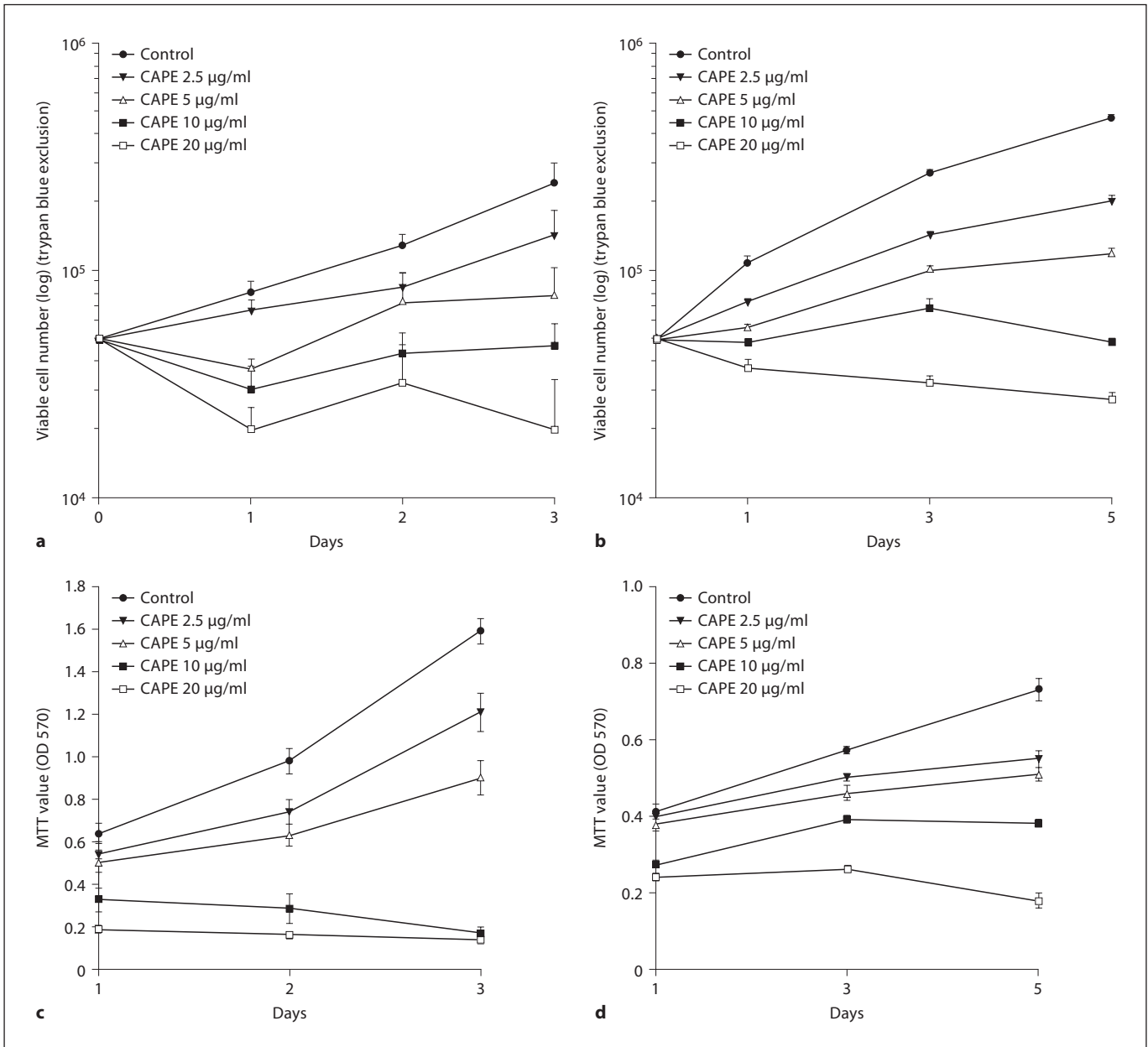


Fig. 1. Cell viability of BxPC-3 cells incubated with different concentration of caffeic acid phenethyl ester (CAPE) (2.5–20 µg/ml). Trypan blue staining (a) and MTT assay (c) were used to assess cell viability. Cell viability of PANC-1 cells incubated with different concentration of CAPE (2.5 to 20 µg/ml). Trypan blue staining (b) and MTT assay (d) were used to assess cell viability. Data from three separate experiments are expressed as mean ± SEM.

Morphological Changes in CAPE-Treated BxPC-3 and PANC-1 Cells

Untreated BxPC-3 cells were round and had a large nucleus, multiple nucleoli, and scanty cytoplasm. The PANC-1 cells were similar in appearance except that the cells were larger and more polygonal than BxPC-3 cells.

PANC-1 cells treated with 10 µg/ml of CAPE for 48 h exhibited condensed chromatin, cytoplasmic membrane blebs, and apoptotic bodies (fig. 2A), with similar changes of condensed and fragmented nuclei seen of treated BxPC-3 cells in Hoechst 33342 stain (fig. 2B).

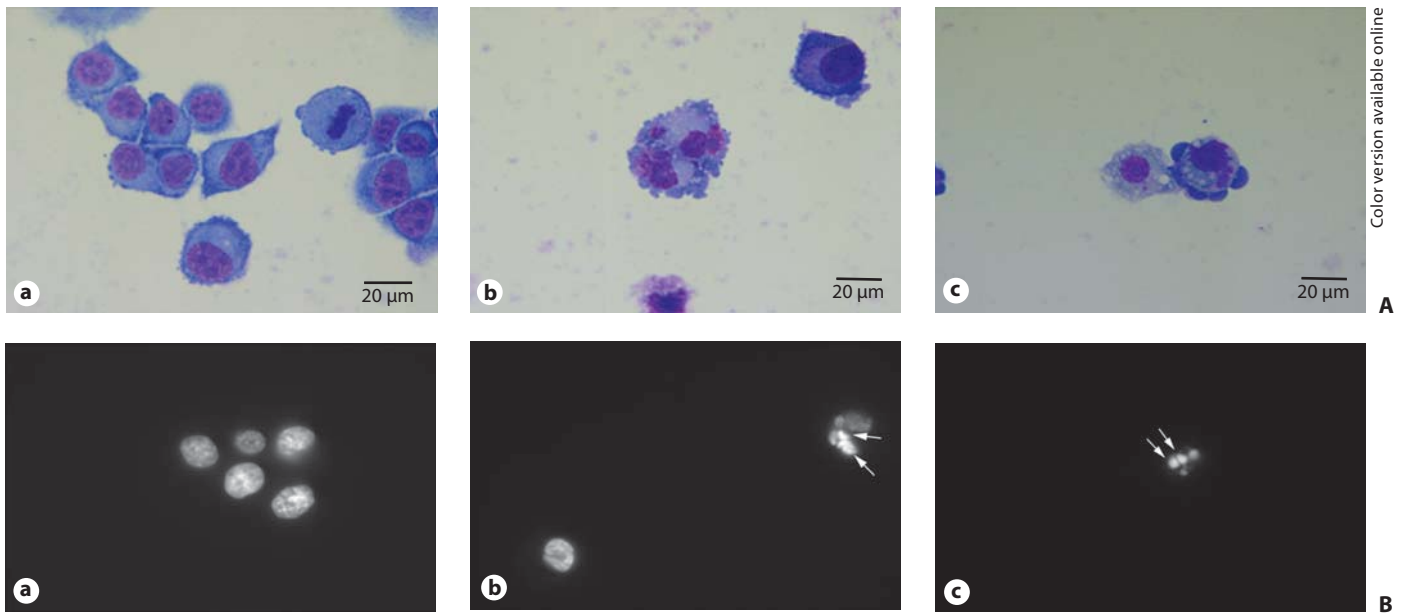


Fig. 2. **A** Morphologic features of PANC-1 cell treated without (**a**) and with CAPE (**b**, 10 $\mu\text{g}/\text{ml}$, **c**, 20 $\mu\text{g}/\text{ml}$) for 48 h (**a** Liu's stain, $\times 1,000$). **B** Morphologic features of BxPC-3 cell treated without (**a**) and with CAPE (**b**, 10 $\mu\text{g}/\text{ml}$, **c**, 20 $\mu\text{g}/\text{ml}$) for 48 h. (**b** Hoechst 33342 stain, $\times 1,000$). Cells undergoing apoptosis have condensed and fragmented nuclei (arrows).

Color version available online

A

B

Effect of CAPE on BxPC-3 Cell-Cycle Distribution
The sub-G1 fraction of BxPC-3 cells increased to $29.0 \pm 8.9\%$ after CAPE treatment in a dose-dependent manner (fig. 3), indicating interference with the normal cell cycle.

Quantitation of Apoptosis by Hoechst Staining and Annexin-V-FITC/PI Double Staining
The total number of early (FITC+/PI-) and late (FITC+/PI+) BxPC-3 apoptotic cells were assessed over time. A concentration of 10 $\mu\text{g}/\text{ml}$ CAPE yielded an apoptotic fraction of $20.1 \pm 2.5\%$ at 48 h (fig. 4a), significantly greater than that seen in untreated cells ($14.0 \pm 1.0\%$). A maximal effect of CAPE treatment was seen at 72 h ($27.0 \pm 3.6\%$, fig. 4b).

We also used DMSO 0.1% v/v as CAPE solvent for repeated experiments. It showed that concentration of 10 $\mu\text{g}/\text{ml}$ CAPE yielded an apoptotic fraction of $11.0 \pm 3.4\%$ at 48 h, significantly greater than that seen in DMSO treated cells ($3.0 \pm 0.8\%$) and untreated cells ($3.3 \pm 0.8\%$) by Annexin-V-FITC/PI double staining. DMSO seemed not to induce apoptosis in BxPC-3 cells. The concentration of 10 $\mu\text{g}/\text{ml}$ CAPE yielded an apoptotic fraction, defined by condensed and fragmented nuclei under obser-

vation by an immunofluorescent microscopy. By Hoechst 33342 staining, it showed that 10.8 and 1.2% at CAPE 10 $\mu\text{g}/\text{ml}$ treated and untreated cells were apoptotic, a data comparable with that by Annexin-V-FITC/PI double staining.

Effect of CAPE on DNA Fragmentation in BxPC-3 Cells
After treatment with 10 to 20 $\mu\text{g}/\text{mL}$ CAPE on BxPC-3 for 2–18 h, no oligonucleosomal DNA fragmentation was found by DNA gel electrophoresis. Gemcitabine (1 μM) was tested as a positive control. It also showed no DNA laddering in BxPC-3 cells (data not shown).

Effect of Pan-Caspase Inhibitor on CAPE-Induced Growth Inhibition
Pretreatment with Z-VAD-fmk (50 μM) reduced the ability of CAPE (10 to 20 $\mu\text{g}/\text{ml}$) to inhibit growth of BxPC-3 cells at 24 h (fig. 5a). This pretreatment also partially blocked the increase in the proportion of sub-G1 cells induced by CAPE (20 $\mu\text{g}/\text{ml}$) from 9.0 ± 2.0 to $1.3 \pm 0.2\%$ at 24 h (fig. 5b). The pan-caspase inhibitor thus partially blocked the inhibition of growth induced by CAPE.

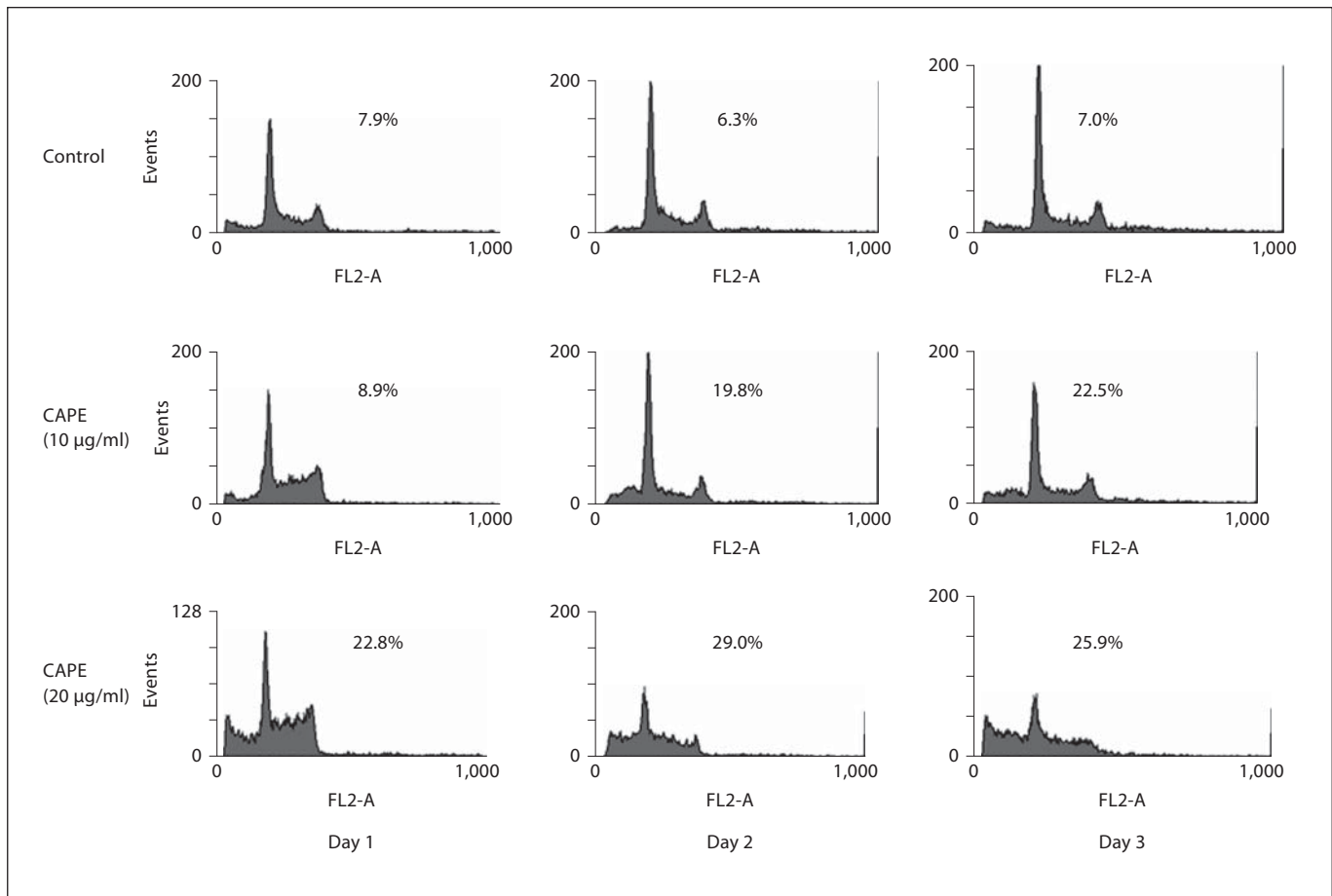


Fig. 3. Cell cycle of BxPC-3 cells treated with CAPE (10 and 20 µg/ml) and untreated controls. There is a significantly higher proportion of the sub-G₁ DNA fraction after treatment for 48 h.

Effect of CAPE on Caspase Substrate Activity Assay

Caspase fluorometric substrate assay showed that caspase-3 and caspase-7 activity was 2.0 ± 0.3 times greater in BxPC-3 cells after treatment with CAPE (10 µg/ml) compared with an untreated control. Changes in the activity of other caspases, including caspase-1, caspase-2, caspase-4, caspase-5, caspase-6, caspase-8, caspase-9 and caspase-10, were not significant.

Loss of Mitochondrial Transmembrane Potential by CAPE Treatment

The mean fluorescence intensity, indicating the mitochondrial transmembrane potential, in untreated cells was 106.1 ± 0.3 . BxPC-3 cells exposed to 10 µg/ml CAPE for 24 and 48 h had a sharp decline in mean fluorescence to 66.2 ± 11.4 and 59.0 ± 10.7 , respectively (fig. 6).

Western Blot Analysis

No change in the expression of Bcl-2, Bax, Smac/Diablo, AIF, or cytochrome C was noted on BxPC-3 after treatment with 10 µg/ml of CAPE for 2–48 h. Treatment with CAPE (10 µg/ml) induced PARP cleavage after 16 h (fig. 7).

Discussion

In this study, the concentrations inducing 50% inhibition of viability in BxPC-3 and PANC-1 cell were relatively low. In concentrations similar to those used in our study, CAPE has been reported to have selective cytotoxicity for cancer cells, to some extent sparing human umbilical vein epithelial cells, lung fibroblast WI-38 cells [30], and buccal mucosa fibroblasts [31]. In an in vivo

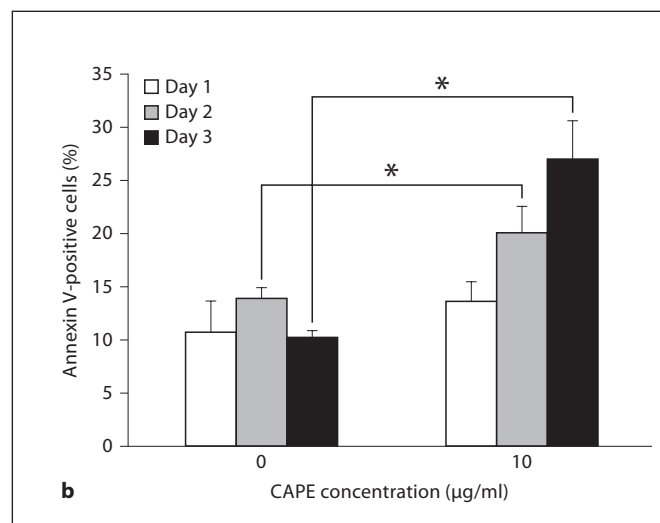
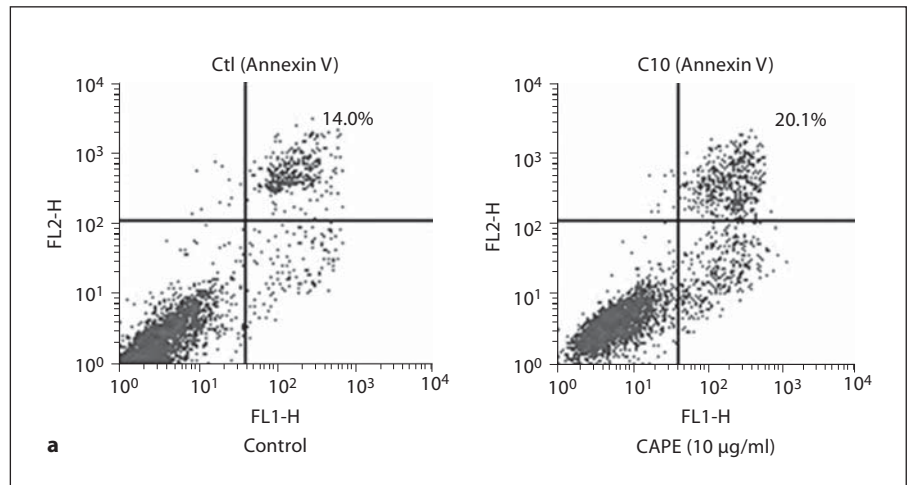


Fig. 4. a Annexin V fluorescent staining of cells indicating apoptotic percentage of BxPC-3 cells without (left, 14.0%) and with CAPE 10 µg/ml (right, 20.1%) for 48 h. **b** Flow cytometric detection of time-dependent induction of apoptosis on BxPC-3 cells by CAPE 10 µg/ml. Data from three separate experiments are expressed as mean ± SEM. * p < 0.05.

study, CAPE sensitized CT26 cells to ionizing radiation in BALB/c mouse without toxicity to bone marrow, liver, and kidney [32]. These findings suggest that CAPE's antitumor effects may be relatively specific to the malignant cells, which would be an improvement on currently available chemotherapy. Cytotoxic agents such as gemcitabine or 5-fluorouracil, for example, are myelosuppressive and thus prone to cause life-threatening neutropenia, anemia, or thrombocytopenia. In this study, we also demonstrated that CAPE induces apoptosis in human pancreatic cancer BxPC-3 cells in association with caspase-3 and caspase-7 activation and mitochondrial dysfunction, as indicated by a decrease in transmembrane potential.

In many cancers, genetic disturbances of apoptotic signaling pathways have been found that are implicated in tumor development and progression. The presence of

point mutations in codon 12 of the K-ras oncogene has been detected up to 83% of human pancreatic cancers [33]. Ras proteins modulate active guanosine triphosphate-bound and inactive guanosine diphosphate-bound states to mediate signal transduction pathways stimulating cell growth and survival. PANC-1 cells carry a K-ras mutation [34], while BxPC-3 cells possess a wild-type ras. In addition to this major genetic disturbance, Bcl2/adenovirus E1B 19-kDa protein interacting protein (BNIP3), a Bcl-2 family proapoptotic protein, has been identified as being expressed at lower levels in drug-resistant pancreatic cancer cell lines [35]. Smad4 (also termed DPC4) appears to be a classical tumor suppressor gene and is mutated or deleted in 50% of pancreatic carcinomas [36]. PANC-1 cells carry wild-type Smad 4 and express a high level of BNIP. BxPC-3 cells possess a mutant Smad 4 and

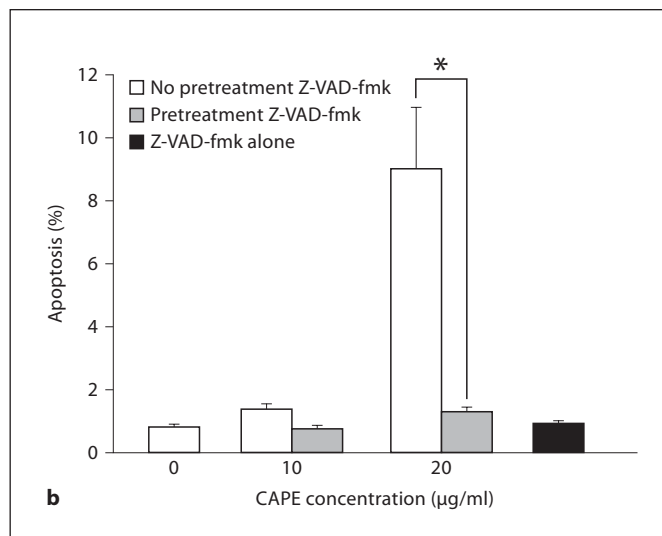
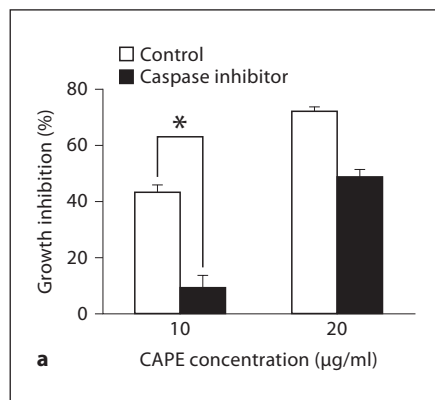
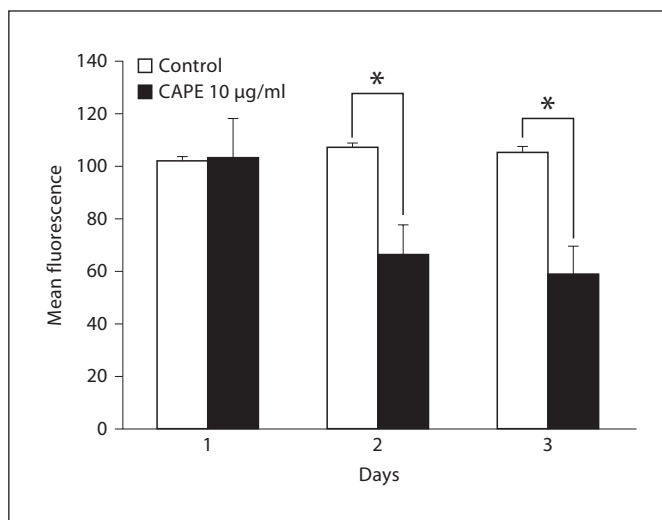


Fig. 5. a Inhibition of growth of BxPC-3 cells by CAPE (10 and 20 µg/ml) at 24 h with or without pretreatment for 2 h with Z-VAD-fmk (50 µM). Data from three separate experiments are expressed as mean ± SEM. * $p < 0.05$. **b** Effect on apoptotic percentage by annexin-PI double staining of BxPC-3 cells after treatment with CAPE (10 and 20 µg/ml) at 24 h with or without pretreatment for 2 h Z-VAD-fmk (50 µM). Data from three separate experiments are expressed as mean ± SEM. * $p < 0.05$.

Fig. 6. Mitochondrial transmembrane potential of BxPC-3 cells treated with CAPE 10 µg/ml. Data from three separate experiments are expressed as mean ± SEM. * $p < 0.05$.



have weak BNIP expression. These differences in gene mutation and expression in the two cell lines, however, did not diminish the ability of CAPE to inhibit their growth. It therefore appears that the effects of CAPE are independent of K-ras or Smad mutations and of BNIP-3 expression. These findings suggest that CAPE might be a useful agent for treating even the most resistant pancreatic cancers, tumors with high numbers of mutations.

In this study, 10 µg/ml of CAPE was more effective than 1 µM of gemcitabine in inhibiting the viability of PANC-1 cells (74.3 ± 2.9 vs. $49.3 \pm 3.8\%$). Multidrug resistance often develops in the course of cancer therapy. This resistance is characterized by overexpression of the membrane efflux pumps P-glycoprotein or multidrug resistance-associated protein. One of the latter proteins,

which confers relative resistance to gemcitabine, is known to be overexpressed by PANC-1 cells [37]. Our results suggest that CAPE might be useful in treating pancreatic cancer resistant to gemcitabine.

The observation that pretreatment with Z-VAD-fmk partially blocked inhibition of growth and induction of apoptosis provides evidence for the involvement of caspases in CAPE's mechanism of action. Caspases, prominent players in apoptosis, belong to a family of cysteine proteases. All caspases are expressed as proenzymes; activation involves a cascade of proteolytic cleavage. Current classification divides caspases into two classes: initiators and effectors. Caspase-8 and caspase-9 are the major initiators and caspase-3 and caspase-7 are the major effectors. We found that CAPE increased caspase-3/cas-

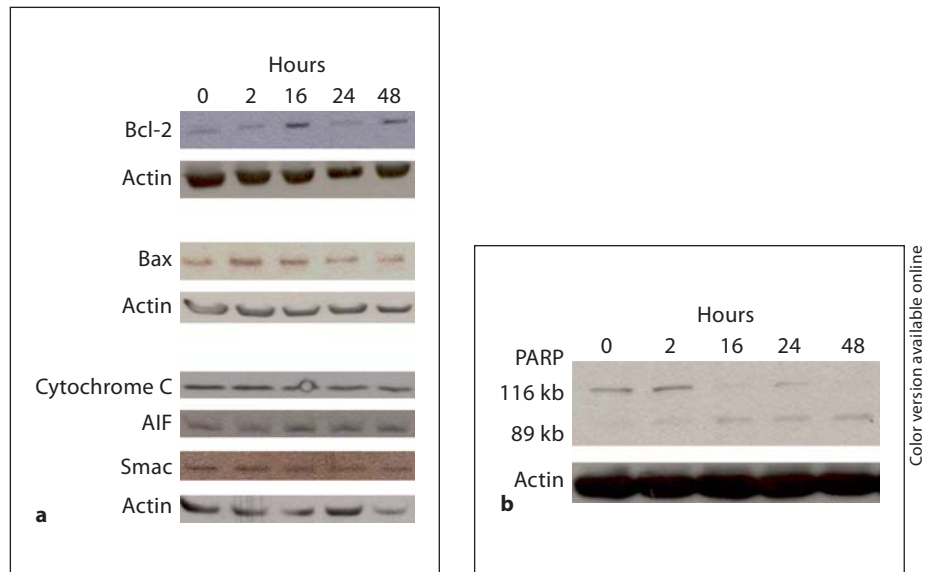


Fig. 7. a Expression of Bcl-2, Bax, Smac/Diablo, AIF and cytochrome C on BxPC-3 after treatment with 10 μg/ml of CAPE for 2–48 h. **b** Expression and processing of PARP on BxPC-3 after treatment with 10 μg/ml of CAPE for 2–48 h.

pase-7 activity 2.0 ± 0.3 times in BxPC-3 without changing the expression of the other caspases we looked at. Cleavage of PARP is one of the detectable proteolytic events following caspase activation, primarily by caspase-3 and caspase-7. We found that PARP was cleavage in CAPE-treated BxPC-3 cells by Western blotting. Thus the effector caspase-3/caspase-7 apparently plays a role in CAPE-induced apoptosis.

Apoptosis can be initiated by two alternative pathways: In type I cells the amount of initiator caspases is sufficient to induce executioner caspases and directly achieve apoptosis. In type II cells, on the other hand, the apoptosis-enhancing effect of mitochondria is necessary to induce the full apoptotic phenotype [38]. The induction of the permeability transition pore which causes mitochondrial depolarization and cytosolic calcium release, results in activation of caspase and following apoptosis [39]. We found that CAPE induced mitochondrial dysfunction, as demonstrated by loss of mitochondrial transmembrane potential. This indicates that, for BxPC-3 cells at least, CAPE appears to function via a type II-cell pathway.

DNA fragmentation is a two-step process in which DNA is first cleaved into 50- to 300-kb fragments and then degraded into smaller oligonucleosomal fragments. In our study, CAPE did not cause DNA fragmentation in BxPC-3 cells. In fact, BxPC-3 cells have never been found to develop obvious DNA fragmentation on agarose gel electrophoresis in response to chemotherapeutic agents. It has been reported that other cells, for example some

neuroblastoma cell lines, also do not develop DNA fragmentation. It is also possible that a defect in death signaling mediates the resistance of BxPC-3 cells to classical apoptosis with DNA fragmentation. Gukovskaya and Pandolfi [38] found a diminished effect of cytochrome c to be one mechanism that prevents sufficient activation of effector caspases. This suggests that pancreatic cancer cells need much greater concentrations than normal of cytochrome c to activate the caspase system. It may be that the resistance to DNA fragmentation in our study was due to insufficient expression of apoptotic signals such as cytochrome c, as demonstrated by its unchanged expression on Western blot.

CAPE at a concentration of 10 μg/ml caused marked inhibition of viability ($80.4 \pm 4.1\%$) of BxPC-3 at 72 h. However, this concentration of CAPE only achieved an apoptotic fraction of $27.0 \pm 3.6\%$ at 72 h, a much lower fraction than in our previous observation of HL-60 cells (66.7%) and A549 cells (67.0%). The latter two results were both achieved with a lower dose of CAPE (6 μg/ml for 72 h). This suggests that the death of BxPC-3 cells might be mediated mainly through apoptosis and with some extent, through the other types of cell death [40].

In conclusion, CAPE inhibits the growth of human pancreatic cancer PANC-1 and BxPC-3 cells. While the complete mechanism is not yet clear, this inhibition at least involves apoptosis via activation of caspase-3 and caspase-7 and perturbation of the mitochondrial transmembrane potential. Much work remains to be done before it is known whether a substance such as CAPE will

be effective clinically. However, preliminary in vitro studies such as this one help to shed light on mechanisms that may eventually be involved in successful treatment of pancreatic cancer.

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