Pancratistatin Selectively Targets Cancer Cell Mitochondria and Reduces Growth of Human Colon Tumor Xenografts

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Abstract

The naturally occurring Amaryllidaceae alkaloid pancratistatin exhibits potent apoptotic activity against a large panel of cancer cells lines and has an insignificant effect on noncancerous cell lines, although with an elusive cellular target. Many current chemotherapeutics induce apoptosis via genotoxic mechanisms and thus have low selectivity. The observed selectivity of pancratistatin for cancer cells promoted us to consider the hypothesis that this alkaloid targets cancer cell mitochondria rather than DNA or its replicative machinery. In this study, we report that pancratistatin decreased mitochondrial membrane potential and induced apoptotic nuclear morphology in p53-mutant (HT-29) and wild-type p53 (HCT116) colorectal carcinoma cell lines, but not in noncancerous colon fibroblast (CCD-18Co) cells. Interestingly, pancratistatin was found to be ineffective against mtDNA-depleted (p53) cancer cells. Moreover, pancratistatin induced cell death in a manner independent of Bax and caspase activation, and did not alter β-tubulin polymerization rate nor cause double-stranded DNA breaks. For the first time we report the efficacy of pancratistatin in vivo against human colorectal adenocarcinoma xenografts. Intratumor administration of pancratistatin (3 mg/kg) caused significant reduction in the growth of subcutaneous HT-29 tumors in Nu/Nu mice (n = 6), with no apparent toxicity to the liver or kidneys as indicated by histopathologic analysis and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling. Altogether, this work suggests that pancratistatin may be a novel mitochondria-targeting compound that selectively induces apoptosis in cancer cells and significantly reduces tumor growth. Mol Cancer Ther; 10(1); 57–68. ©2011 AACR.

Introduction

Colorectal cancer is the third most diagnosed cancer worldwide and has a mortality rate of nearly 1 in 2 (1). Current adjuvant chemotherapy of metastasized colorectal cancer includes DNA machinery-targeting drugs irinotecan, oxaliplatin, leucovorin, and/or 5-fluorouracil (1). More than 50% of chemotherapy agents currently in use are natural products or derived directly from natural sources, and recently there has been increasing interest in natural products as potential sources for novel therapeutics (2, 3). The findings presented in this article suggest that the natural alkaloid pancratistatin may act directly on the mitochondria to induce apoptosis selectively in human colorectal adenocarcinoma cells.

Pancratistatin is an Amaryllidaceae alkaloid isolated from the bulb of the Hymenocallis littoralis species of spider lily. Numerous Amaryllidaceae alkaloids have been isolated and have diverse bioactivities, a few with potential medicinal properties (4). We have recently shown that pancratistatin induces apoptosis in several cancer cell lines and in ex vivo models of leukemia, with minimal effect on the growth and survival of noncancerous counterpart cells (5–7). We have previously reported that pancratistatin promotes reactive oxygen species (ROS) generation and mitochondrial depolarization, leading to apoptosis in breast carcinoma cells independent of estrogen receptor status (8, 9).

Apoptosis is a cell death program that can be induced by either the extrinsic or intrinsic pathways, although extensive cross-talk exists between them (10). Classical genotoxic chemotherapeutic agents cause intrinsic apoptosis by inducing extreme stress to the DNA and its repair mechanisms, and are also toxic to noncancerous cells (11). Development of targeted therapeutics led to increased selectivity, but these options are often effective against only a small subset of cancers. Recently, a number of naturally derived mitochondria-directed small molecules, referred to as mitocans, have shown promise as selective anticancer agents (12, 13). Mitochondrial perturbation, either directly by small molecules or indirectly by activation of proapoptotic proteins, causes release of cytotoxic components such as cytochrome c, which leads to caspase activation, and ultimately apoptosis (14).

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Mitochondria-targeted compounds may be able to induce apoptosis independent of p53—a frequently mutated tumor suppressor in human carcinomas (15).

In response to cellular stress, multifunctional p53 translocation factor induces the intrinsic pathway of apoptosis by activating specific proapoptotic BH3-only proteins of the Bcl-2 family (16). Activated BH3-only proteins further activate multidomain proapoptotic proteins Bax and/or Bak, which localize to the mitochondria and form oligomers that cause mitochondrial outer membrane permeabilization (MOMP), leading to apoptosis (13). In this way, p53 plays a very important role as a potent tumor suppressor protein. Loss-of-function mutations in p53 correlate to aggressive, chemoresistant tumors, especially in patients with colorectal cancer (10). In this study, the p53-negative HT-29 colorectal adenocarcinoma cell line was compared with the wild-type p53 HCT116 colorectal carcinoma cell line to determine the efficacy of pancratistatin in both systems (10).

Here we report the novel finding that pancratistatin induces apoptosis in HT29 and HCT116 colon carcinoma cell lines in vitro and reduces growth of HT-29 xenografts in vivo. This study suggests that cell death induced by pancratistatin may be dependent on a component of the mitochondrial respiratory chain (MRC). For the first time, we show that pancratistatin significantly reduces growth of p53-mutant HT-29 xenografts in nude mice and importantly, pancratistatin does not cause cell death in noncancerous CCD-18Co colon fibroblasts. Our findings suggest that pancratistatin selectively targets cancer cell mitochondria to induce apoptosis, with minimal toxicity to noncancerous cells.

Materials and Methods

**Chemicals**

Media, gentamycin, Hoechst 33342 dye, JC-1 dye, Amplex Red, Annexin-V AlexaFluor 488, and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit were purchased from Invitrogen (GIBCO). Cell proliferation reagent WST-1 was purchased from Roche. A water-soluble formulation of Coenzyme Q10 (ws-CoQ10) and a generous gift from Dr. J. McNulty. All other chemicals were purchased from Bio-Rad. Pancratistatin (Fig. 1A) was isolated from H. littoralis as previously described (17) and provided by Dr. J. McNulty. All other chemicals were purchased from Sigma-Aldrich.

**Cell lines, culture conditions, and assessment of apoptosis**

Normal colon fibroblasts (CCD-18Co) and human colorectal adenocarcinoma (HT-29, HCT116) cells were purchased from American Type Culture Collection. Cells were passaged for less than 6 months and no authentication of cell lines was performed by the authors. HT-29 cells were grown in McCoy’s 5A media and CCD-18Co cells were grown in Eagle’s minimum essential medium (MEM); both were supplemented as recommended by ATCC. Parental stock glioblastoma (U87MG) and the counterpart mtDNA-depleted cell line (U87MGp0) were a generous gift from Dr. M. Sikorska at NRC (18). These cells were grown in MEM supplemented with 10% fetal bovine serum and 10 μmol/L gentamycin. Human neuroblastoma (SH-SY5Y) cells transfected with single-domain antibodies against Bax protein were established and maintained as previously described (19). All cells were maintained at 5% CO2 and 37°C. Cells were treated as indicated with 1 μmol/L pancratistatin, predetermined as the effective concentration (7, 20). Nuclear morphology was evaluated by fluorescence microscopy after staining with Hoechst 33342 dye. Percent condensed nuclei (indicative of apoptosis) was calculated as [number of cells with condensed, brightly stained nuclei / total number of cells] × 100 with a minimum 5 fields with 100 cells/field counted.

**Cytotoxicity assays**

Cytotoxicity was measured using the WST-1 colorimetric assay following the manufacturer’s protocol. Briefly, cells were seeded at 2,000 to 5,000 cells/well in 96-well tissue culture plates, and allowed to adhere overnight and treated as indicated. Cells were then incubated with WST-1 for 4 hours at 37°C. Absorbance was measured at 450 nm on a VICTOR2 microplate reader. Absorbance is directly proportional to the number of living cells in culture. Percent viability was calculated based on average absorbance of untreated cells. To monitor the effect of pancratistatin on cell growth, cells were counted using the trypan blue exclusion assay.

**Mitochondrial functionality assays**

Mitochondrial membrane potential (MMP) was observed using cell-permeable JC-1 dye (1 μmol/L) as previously described (8). To detect generation of ROS, cells were treated with pancratistatin for the indicated time, trypsinized, and incubated with H2DCFDA (1 μmol/L) for 45 minutes at 37°C in an opaque 96-well plate. Fluorescence was measured at Ex. 513 nm and Em. 530 nm using a SpectraMax GeminiXS spectrofluorometer. Change in ROS generation was calculated as the fold difference from control [relative fluorescence units (RFU)] per 105 cells; cells were counted using the trypan blue exclusion assay. Cytotoxicity and ROS generation were used to analyze the effect of antioxidants on pancratistatin treatment. Cells were cotreated with pancratistatin (1 μmol/L) and either N-acetyl cysteine (NAC; 5 mmol/L) or ws-CoQ10 (50 μg/mL) for 48 or 72 hours.
Mitochondrial isolation and Western blotting

Cells treated with pancratistatin were washed with ice-cold PBS and the pellet was resuspended in cold mitochondrial isolation buffer (75 mmol/L sucrose, 20 mmol/L HEPES, 225 mmol/L Mannitol, 0.5 mmol/L EDTA, pH 7.2) and then disrupted by homogenization. Nonlysed cells/nuclei were pelleted by centrifugation at 500 g for 15 minutes at 4°C. The supernatant was further centrifuged at 4°C and 10,000 g for 10 minutes twice. The resulting pellet was designated as the mitochondrial fraction and the supernatant as the cytosolic fraction. Protein samples (20 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated primary antibody (1:1,000) overnight at 4°C. Membranes were then incubated with peroxidise-conjugated secondary antibody (1:1,000) for 1 hour at 25°C and visualized using enhanced chemiluminescence reagent. Equal loading of the protein samples was confirmed by parallel Western blots for β-actin or SDH-A. Images were digitized and band intensity was quantified using NIH Image J software.

β-Tubulin polymerization activity assay

To determine whether pancratistatin affects the rate of β-tubulin polymerization/depolymerization compared with known polymerizing agent paclitaxel, the HTS-Tubulin Polymerization Assay kit (Cytoskeleton Inc.) was utilized as described by the manufacturer and elsewhere (21). Microtubule assembly was monitored by spectrophotometry as a change in absorbance at 340 nm every minute for 60 minutes at 37°C using a SpectraMax M5e microplate reader.

Histone H2AX phosphorylation

To detect double-strand DNA breaks, cells were grown on coverslips, treated with pancratistatin for 6 hours, and then processed for immunohistochemical analysis of histone H2AX phosphorylation on Ser-139, as previously described (7).

In Vivo xenograft models

Six-week-old male homozygous CD-1 nude mice (25–30 g) were obtained from Charles River Laboratories. Animals were housed in constant laboratory conditions of a 12-hour light/dark cycle at 21°C in accordance with institutional animal protocols (University of Windsor Research Ethics Board—AUPP05). Prior to injection, HT-29 cells were washed with PBS and centrifuged at 500 × g for 5 minutes; cell pellets were suspended in aliquots of 200 μL PBS (4 × 10^6 cells per mouse). The cell suspension was injected s.c. in the right hind flank of each mouse. Tumors were allowed to grow to approximately 50 mm^3 and thereafter animals were randomized into treatment groups of 6 mice each (day 14 of study). Animals were treated intratumorally (i.t.)
with vehicle (5 μL Me₂SO in PBS) or pancratistatin (3 mg/kg) twice a week for 5 weeks (22, 23). Tumors were assessed at each treatment by measuring length and width using standard calipers; tumor volume was calculated using the formula: \( \pi/6 \times \text{length} \times \text{width}^2 \). In parallel, tumor-free mice were treated intraperitoneally (i.p.) twice a week for 2 months with vehicle or pancratistatin for toxicity studies. All animals were assessed for weight loss twice weekly for the duration of the experiment.

Hematoxylin and eosin staining and immunohistochemical analysis

One week after the last treatment, the tumor, liver, and kidneys were collected from all animals and fixed in 10% formalin in PBS for up to 24 hours, then moved to 70% ethanol. Sections of 8 μm thickness were sliced from paraffin-embedded tissues and placed on Superfrost/Plus microscope slides (Fisher Scientific) for immunohistochemical and histopathologic analysis. Slides were deparaffinized and hydrated for TUNEL assay.

Results

Pancratistatin selectively induces cell death in colon cancer cells

We determined the effect of pancratistatin (see Fig. 1A for chemical structure) on the viability of HT29 and HCT116 human colorectal adenocarcinoma cells, and noncancerous human colon fibroblast CCD-18Co cells. Nuclear morphology of HT-29 and HCT116 cells after 72 hours of pancratistatin exposure revealed brightly stained condensed nuclei characteristic of cells undergoing apoptosis, in contrast to CCD-18Co nuclear morphology, which was unchanged (Fig. 1B). Viability of both colon cancer cell lines was significantly reduced after 72-hour exposure to pancratistatin in a concentration-dependent manner, with an IC₅₀ of approximately 100 nmol/L for both cell types (Fig. 1A). By comparison, 100 nmol/L pancratistatin was significantly less toxic to noncancerous CCD-18Co, and the IC₅₀ for this noncancerous cell line was determined to be 10 μmol/L (Fig. 1C). We have previously reported the selective anticancer activity of pancratistatin using human cancerous and noncancerous breast cell lines, and human leukemic and noncancerous peripheral blood mononuclear cells (6, 9). As shown in Fig. 1D, growth of HT-29 cells was dramatically reduced when treated with pancratistatin, especially when pancratistatin was added again after 72-hour initial exposure. Moreover, HT29 cells remaining after 144-hour pancratistatin single treatment were unable to proliferate when given fresh drug-free media and cultured for up to 72 hours (Fig. 1D). A similar effect was also observed in HCT116 cells (Supplementary Fig. S1). These results indicated that pancratistatin decreased survival of human colorectal carcinoma cells, irrespective of p53-status, with minimal toxicity to noncancerous colon fibroblasts.

Pancratistatin destabilizes cancer cell mitochondria

Dissipation of the MMP, increased generation of ROS, and release of proapoptotic factors such as cytochrome c are characteristic features of mitochondria-mediated apoptosis. Thus, we monitored the occurrence of these phenomena in HT-29, HCT116, and CCD-18Co cell lines following pancratistatin treatment. First, the effect of pancratistatin on MMP using JC-1 fluorescence microscopy was observed. HT-29 cells treated with pancratistatin had a reduction in JC-1 aggregation compared with Me₂SO-treated (control) cells, as observed by a decrease in fluorescence. HCT116 cells also displayed reduced aggregation of JC-1 dye compared with control, indicating loss of MMP. Importantly, there was no evidence of MMP collapse in noncancerous CCD-18Co cells treated with the same concentration of pancratistatin for 96 hours (Fig. 2A). This finding suggested that pancratistatin specifically targets cancer cell mitochondria to cause MMP collapse and subsequent cell death, without affecting the potential of noncancerous mitochondria. Next, we tested the degree of ROS generation in HT-29 and HCT116 cells following exposure to pancratistatin. Overproduction of ROS can trigger intrinsically mediated apoptosis and has recently been described as a powerful method to induce cancer cell death in a selective manner (12). Indeed, in both cell lines there was a significant increase in ROS production that correlated with increased pancratistatin incubation time (Fig. 2B). Last, we tested for the release of the proapoptotic protein cytochrome c from the mitochondria to the cytosol in pancratistatin-treated HT-29 cells. In accordance with pancratistatin-mediated MMP collapse, Western blot analysis showed release of cytochrome c into the cytosolic fraction of cells treated with pancratistatin compared with untreated control cells (Fig. 2C). A similar result was observed in HCT116 cells (Supplementary Fig. S2A).

To determine the effect of antioxidants on pancratistatin-induced ROS generation and cell death, HT-29 cells were cotreated with pancratistatin and NAC for 48 and 72 hours. As expected, cotreatment with NAC reduced ROS generation by approximately 2-fold compared with pancratistatin alone; however, NAC failed to protect cells from pancratistatin-induced apoptosis (Fig. 2D). Furthermore, cotreatment of pancratistatin with the antioxidant CoQ₁₀ also failed to protect HT-29 cells from reduced cell viability due to 72-hour pancratistatin treatment (Supplementary Fig. S2B). Together, these results indicated that pancratistatin caused collapse of MMP that was associated with release of cytochrome c and increased ROS generation.

Mitochondrial DNA-deficient ρ⁰ cells are resistant to pancratistatin-induced apoptosis

To firmly establish that mitochondria are involved in pancratistatin-induced cell death, we used the Rho-0 (ρ⁰) variant of U87MG glioblastoma cells in parallel with wild-type “parental” U87MG cells. Rho-0 cells depend on ATP derived from anaerobic glycolysis for survival;
these cells lack the machinery required for oxidative phosphorylation and thus cannot generate ROS (24). We then aimed to determine whether pancratistatin can induce apoptosis in either the parental or \( p^0 \) U87MG cancer cells. As anticipated, we found that \( p^0 \) cells were resistant to 1 \( \mu \text{mol/L} \) pancratistatin compared with wild-type cells, which had nuclear and cellular morphology characteristic of apoptosis (Fig. 3A).
Exposure to pancratistatin for 120 hours resulted in a significant (50%) increase in apoptosis of wild-type cells over to \( \rho^0 \) cells (Fig. 3B). This finding suggested that pancratistatin may target a component of MRC complexes to selectively induce apoptosis in cancer cells.

**Bax inhibition does not attenuate pancratistatin-induced apoptosis**

The Bcl-2 family of anti- and proapoptotic proteins is involved in the regulation of the intrinsic pathway of apoptosis. Activation of proapoptotic Bax protein leads to permeabilization of the mitochondrial outer membrane and subsequent cell death. To investigate whether mitochondrial destabilization caused by pancratistatin is mediated by Bax, we utilized intracellularly expressed anti-Bax single-domain antibodies (sdAb), also termed intrabodies, previously established in our laboratory (19). Stable transfection with 1 of 3 anti-Bax intrabodies (sdAb-1, -2, -4) into human neuroblastoma (SH-SYSY) cells effectively blocks the proapoptotic function of Bax protein in vivo, and has been shown to prevent oxidative stress-induced apoptosis (19). As shown in Fig. 3C, Bax inhibition was unable to protect SH-SYSY cells from pancratistatin-induced apoptosis. This novel finding suggested that pancratistatin acts independent of Bax function to induce cell death in cancer cells.

Furthermore, we studied the expression and localization of endogenous Bcl-2 family proteins in HT-29 and HCT116 cell lines. Interestingly, pancratistatin treatment caused increased mitochondrial localization of Bax protein in wild-type p53 HCT116 cells compared with control (Fig. 3D). In contrast, pancratistatin caused no change in localization of Bax in p53-negative HT-29 cells, but instead caused increased expression of the protein in the cytosol (Fig. 3D). The significance of the differential response to pancratistatin treatment between cell lines is unknown at this time, and may be due to p53-mediated effects. In both cell lines, Bcl-2...
protein was largely contained to the mitochondrial fraction, and pancratistatin treatment resulted in an increased proportion of cytosolic Bcl-2 significantly in HCT116 cells (Fig. 3D). Taken together, these findings suggested that although pancratistatin may alter expression and localization of Bcl-2 family proteins, the proapoptotic function of Bax is not required for cell death induced by pancratistatin.

Pancratistatin induces apoptosis independent of caspase activation

To further characterize the mechanism of action of pancratistatin, we treated HT-29 cells in the presence of a cell-permeable, irreversible pan-caspase inhibitor (z-VAD-fmk) to determine whether pancratistatin-induced apoptosis is caspase dependent. After 72-hour exposure to pancratistatin in the presence or absence of z-VAD-fmk, cells were monitored for apoptotic nuclear morphology and viability using Hoescht dye and WST-1 assay. Unexpectedly, we found that pancratistatin effectively induced apoptotic nuclear morphology in HT-29 cells independent of caspase activity (Fig. 4A). Indeed, pancratistatin retained the ability to significantly reduce cell viability of HT-29 cells when cotreated with a pan-caspase inhibitor at various concentrations (Fig. 4B). These results suggested that pancratistatin induced cell death independent of caspase activation. To further investigate this hypothesis, the effect of pancratistatin on caspase-independent proapoptotic proteins was analyzed. Figure 4C depicts the release of AIF and Endo G to the cytosol following treatment with pancratistatin in HT-29 cells. Similar results were observed in HCT116 cells (not shown). These findings suggested that pancratistatin-induced cell death may occur via caspase-independent mechanisms.

Pancratistatin does not interfere with microtubules or induce DNA damage as a primary mechanism of action

To determine whether pancratistatin induces apoptosis in a manner similar to other plant alkaloids, namely the microtubule interfering compounds vincristine and
paclitaxel, we tested the ability of pancratistatin to alter the polymerization rate of β-tubulin. In a direct comparison between pancratistatin and paclitaxel, we found that pancratistatin had no effect on β-tubulin polymerization rate compared with the expected rate increase caused by paclitaxel (Supplementary Fig. S3A). This result indicated that pancratistatin does not interfere with microtubules to illicit a cell death response.

We have previously reported that, unlike genotoxic chemotherapeutics such as etoposide, pancratistatin does not damage DNA as a primary mechanism of action, as evidenced by comet assay and histone H2AX phosphorylation (7). Here we report a similar negative result that 6-hour pancratistatin treatment did not cause double-strand DNA breaks in HT-29 and HCT116 cells. At this early time point there were no visible foci, which are representative of phosphorylation of histone H2AX in response to DNA damage (Supplementary Fig. S3B). This result supported our earlier findings that pancratistatin is a nongenotoxic compound.

**Pancratistatin administration reduces growth of HT-29 xenografts**

To test the *in vivo* significance of our cellular observations, we determined the effect of pancratistatin administration on HT-29 xenograft growth in nude mice. Pancratistatin was administered i.t. twice a week at 3 mg/kg into animals bearing HT-29 xenografts. After 5 weeks of treatment, the average tumor volume in pancratistatin-treated mice (18.0 ± 0.6 mm³) was approximately 15-fold lower than the average tumor volume in control mice (249.5 ± 170.2 mm³). Furthermore, after the 10 treatments there was a approximately 2.5-fold reduction in tumor volume in pancratistatin-treated animals compared with the average tumor volume in all animals (45.9 ± 5.4 mm³) on day 1 of treatment (Fig. 5B and C). These results indicated that pancratistatin significantly reduces growth of HT-29 xenografts in nude mice without causing any observable side effects. To determine whether pancratistatin-mediated inhibition of HT-29 xenograft growth *in vivo* was accompanied by increased apoptosis, tumor tissues from control and pancratistatin-treated animals were processed for TUNEL assay. Compared to control, the pancratistatin-treated tumors had an increased amount of TUNEL-positive cells, which corresponded with brighter DAPI staining (Fig. 5D). The average body weight of control or pancratistatin-treated mice did not vary significantly throughout the study (Fig. 5A). There were no observable signs of distress such as impaired movement, areas of redness or swelling, or change in behavior in pancratistatin-treated animals compared with control.
To further assess whether pancratistatin had toxic effects on noncancerous tissues, the liver and kidneys from animals treated with control or i.p. injection of pancratistatin were processed for histologic analysis. Hematoxylin and eosin (H&E) staining of the liver and kidneys revealed no gross morphologic difference between treatment groups (Fig. 6A). Tumor sections from control and pancratistatin-treated (i.t.) animals were also prepared for histologic analysis by H&E staining. Figure 6B shows the difference in cellular morphology and tumor organization between the 2 groups. Together, our in vivo data indicated that pancratistatin significantly reduced tumor volume in HT-29 xenograft tumors by inducing apoptosis, without causing weight loss or obvious adverse effects to noncancerous tissues.

**Discussion**

Induction of apoptosis by chemotherapeutic agents is often mediated through p53-dependent mechanisms; however, the majority of human carcinomas have p53 inactivation or mutations. The aim of this study was to investigate the potential anticancer activity of the natural alkaloid pancratistatin against p53-negative (HT-29) and wild-type p53 (HCT116) human colorectal carcinoma cell lines, and to delineate its mechanism of action. This study revealed that pancratistatin has significant apoptotic activity against both cancer cell lines in culture and suppresses growth of HT-29 xenograft tumors. Interestingly, pancratistatin did not elicit an apoptotic response in noncancerous human colon fibroblast (CCD-18Co) cells. This differential sensitivity to pancratistatin has been observed for other cancer cell lines and normal counterparts, both in vitro and ex vivo (6, 8, 9). We have previously shown that pancratistatin does not induce double-strand DNA breaks or comet formation at early time points compared with etoposide and paclitaxel (7, 25). In this report, pancratistatin did not cause double-strand breaks in the DNA of HT-29 or HCT116 cells, nor did it affect microtubule assembly. Instead, pancratistatin-induced cell death was found to be mitochondria-dependent. Pancratistatin caused increased ROS production, collapse of MMP, and cytochrome c release. Furthermore, pancratistatin was found to act independent of p53, Bax, and caspase activation, through the use of p53-negative HT-29 cells, mtDNA-depleted (ρ0) cells, and caspase- and Bax-inhibition assays. Last, pancratistatin treatment caused significant growth reduction of HT-29 tumor xenografts with limited effects on noncancerous tissues.

Collapse of the MMP and release of cytochrome c indicates that the intrinsic pathway of apoptosis is induced by pancratistatin. Once released, cytochrome c complexes with pro-caspase-9 and Apaf-1 to form the apoptosome, which activates caspase-3 resulting in apoptosis (26). Along with cytochrome c, other proapoptotic proteins are released including AIF and Endo G, which can trigger caspase-independent cell death (26, 27). In this report we show that pancratistatin reduced viability of HT-29 cells in the presence of a general caspase inhibitor, and also lead to the release of AIF and Endo G from the mitochondria of these cells (Fig. 4). Our hypothesis that this alkaloid targets the mitochondria and triggers death upstream of caspase activation is bolstered by our result that caspase inhibitors cannot prevent pancratistatin-induced cell death. Complimentary to this study, we have previously reported that pancratistatin induces cell death in caspase-3–deficient estrogen receptor–positive MCF-7 breast cancer cells, and was also effective against estrogen receptor–negative Hs-578-T breast cancer cells (9).

A common mechanistic theme of mitochondriotoxic small molecules is that they act as antagonists of anti-apoptotic Bcl-2 family proteins, or are designed to mimic BH3-only proteins (15, 28). In either case, the result is activation of Bax, which is responsible for MOMP (26). In this study, we utilized intracellularly expressed anti-Bax single-domain antibodies (intrabodies) previously established in our laboratory to examine the effect of functional inactivation of Bax on pancratistatin activity (19). Interestingly, stable expression of anti-Bax intrabodies did not attenuate pancratistatin-induced apoptosis in neuroblastoma cells, suggesting that pancratistatin is neither a Bcl-2 family protein antagonist nor a BH3-mimetic.
type p53 colorectal carcinoma cells, pancratistatin induced mitochondrial localization of Bax, where its proapoptotic function is activated, and increased the proportion of cytosolic Bcl-2, which inactivates the antiapoptotic function of this protein. In contrast, Bax and Bcl-2 subcellular localization was largely unaffected by pancratistatin treatment in p53-negative HT-29 cells. These findings indicate that although pancratistatin alters the locale, and thus activity, of Bcl-2 family proteins, the proapoptotic member Bax is not a direct target of pancratistatin treatment and its function is indispensable for pancratistatin-induced cell death. Possible involvement of the functionally similar proapoptotic Bak protein, however, cannot be ruled out, as it has been shown to compensate for Bax inhibition (15).

Cancer cell mitochondria are prime targets for the development of selective anticancer agents, as they have manipulative differences from normal cell mitochondria that can be exploited (28, 29). Unlike normal cell mitochondria, which utilize oxidative phosphorylation to generate ATP, cancer cells mainly depend on anaerobic glycolysis for energy (Warburg theory; ref. 30). As a result, cancer cells have increased dependence on glucose and their mitochondria are typically hyperpolarized (31). Targeting components of the MRC complexes, which are essentially dormant in cancer cells, should result in selective cancer cell death (29). Compounds shown to inhibit MRC complex activity, such as the natural phytochemicals benzyl- and phenethyl-isothiocyanate, cause increased ROS production and MOMP specifically in cancer cells (32). In this study, we show that pancratistatin treatment increased ROS production, collapse of MMP, and release of cytochrome c in colorectal cancer cells. In this study and in previous reports, we have shown that pancratistatin does not affect MMP nor lead to increased ROS production in noncancerous cells (8). In an attempt to delineate the hierarchy of ROS production in pancratistatin-induced cell death, cotreatment with specific antioxidants or MRC complex inhibitors was performed. Our results demonstrate that the antioxidant NAC effectively quenched pancratistatin-induced ROS production, but it was unable to prevent cell death. Interestingly, cotreatment with the antioxidant CoQ10 did not alter the significant reduction in cell viability following pancratistatin treatment (Supplementary Fig. S2B). These findings indicated that either pancratistatin does not directly compete with compounds, or that oxidative insult is not the primary cause of pancratistatin-induced cell death. Moreover, we report that mtDNA-deficient p0 cancer cells resisted pancratistatin-induced cell death, which indicated that pancratistatin may be targeting a component of MRC complexes, as the parental cell line was sensitive to treatment (18, 32).

In response to cellular stress, Bax is activated either directly by p53 or indirectly by p53-mediated transcriptional regulation of BH3-only proteins Puma and Noxa (30). A potent tumor suppressor, loss-of-function mutations of p53 correlates with chemotherapy and aggressive tumors; p53 mutation is considered to be an early step in the development of colon carcinoma (33). Here we report that pancratistatin effectively induced apoptosis and caspase-independent cell death in human colorectal adenocarcinoma (HT-29) cells that have a loss-of-function mutation of p53. Additionally, wild-type p53 colorectal carcinoma (HCT116) cells were proven sensitive to pancratistatin-induced apoptosis. Importantly, as proof-of-concept, we report that i.t. administration of pancratistatin caused a significant reduction in the growth of HT-29 xenograft tumors in nude mice. The concentration of pancratistatin used in this study (3 mg/kg) is below the previously determined maximum tolerated dose (22). There was no morbidity due to treatment, nor was there drastic variation in activity level or significant weight loss/gain between control and treated animals, indicating low toxicity of pancratistatin in vivo. This observation correlated with histopathology analysis of the liver and kidneys from control and pancratistatin-treated animals having no gross morphologic differences. The antitumor activity of pancratistatin was found to be more potent than sodium pancratistatin 3,4-ocyclic phosphate, reported by Shnyder and colleagues (34) to have antitumor activity at 100 mg/kg. Other Amaryllidaceae alkaloids, including narcilasine and lycorine, have antitumor activity at doses that complement our findings (35, 36).

The structurally minimum pharmacophore required for potent anticancer activity has been defined in the pancratistatin series of alkaloids based on a number of structure-activity correlations that have been conducted with both natural and synthetic analogues (37, 38). The pharmacophore consists of the 2,3,4-triol structural element in ring-C. Although not requirements, the pharmacophoric element is moderated slightly by the incorporation of the C7 phenolic hydroxyl group and, interestingly, through the inclusion of a β-benzoyloxy substituent on the C1 position (37, 39). Because these features are not crucial, they highlight areas where further elaboration is possible on top of the intact pharmacophore. Fully functionalized seco-analogues of both the lactam and cyclitol rings are inactive, indicating that a fully intact tetracyclic core, having the correctly positioned hydroxyl groups in ring-C, is required (40).

In conclusion, the results presented in this article show that pancratistatin selectively induces cell death and reduces growth of human colorectal adenocarcinoma cells in culture and in vivo. Pancratistatin effectively induces cell death independent of Bax and caspase activation by targeting cancer cell mitochondria. We are currently working to further unravel the networks, interactions, and pathways that pancratistatin employs to induce cell death in both p53 wild-type and mutated cell lines. Overall, mitochondriotoxic compounds show great promise as specific and potent anticancer agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.
Author Contributions

C. Griffin contributed to study design, performed and analyzed experiments, and prepared the paper; A. Karmik performed and analyzed experiments; S. Pandey contributed to study design, analyzed experiments, and helped prepare the paper; J. McNulty provided pancratistatin, contributed to study design, and helped prepare the paper.

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Molecular Cancer Therapeutics

Pancreatistatin Selectively Targets Cancer Cell Mitochondria and Reduces Growth of Human Colon Tumor Xenografts

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