

Cucurbitacin B Induces Apoptosis by Inhibition of the *JAK/STAT* Pathway and Potentiates Antiproliferative Effects of Gemcitabine on Pancreatic Cancer Cells

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Abstract

Pancreatic cancer is an aggressive malignancy that is generally refractory to chemotherapy, thus posing experimental and clinical challenges. In this study, the antiproliferative effect of the triterpenoid compound cucurbitacin B was tested *in vitro* and *in vivo* against human pancreatic cancer cells. Dose-response studies showed that the drug inhibited 50% growth of seven pancreatic cancer cell lines at 10^{-7} mol/L, whereas clonogenic growth was significantly inhibited at 5×10^{-8} mol/L. Cucurbitacin B caused dose- and time-dependent G₂-M-phase arrest and apoptosis of pancreatic cancer cells. This was associated with inhibition of activated *JAK2*, *STAT3*, and *STAT5*, increased level of *p21^{WAF1}* even in cells with nonfunctional *p53*, and decrease of expression of *cyclin A*, *cyclin B1*, and *Bcl-XL* with subsequent activation of the *caspase* cascade. Interestingly, the combination of cucurbitacin B and gemcitabine synergistically potentiated the antiproliferative effects of gemcitabine on pancreatic cancer cells. Moreover, cucurbitacin B decreased the volume of pancreatic tumor xenografts in athymic nude mice by 69.2% ($P < 0.01$) compared with controls without noticeable drug toxicities. *In vivo* activation of *JAK2/STAT3* was inhibited and expression of *Bcl-XL* was decreased, whereas *caspase-3* and *caspase-9* were up-regulated in tumors of drug-treated mice. In conclusion, we showed for the first time that cucurbitacin B has profound *in vitro* and *in vivo* antiproliferative effects against human pancreatic cancer cells, and the compound may potentiate the antiproliferative effect of the chemotherapeutic agent gemcitabine. Further clinical studies are necessary to confirm our findings in patients with pancreatic cancer. [Cancer Res 2009;69(14):5876–84]

Introduction

Pancreatic cancer is a severe health problem and a leading cause of cancer-related deaths (1). Despite recent advances in the understanding of the molecular biology of pancreatic cancer, the

annual incidence still is almost identical to the mortality rate. The low survival rate is due to insensitivity of pancreatic cancer to most therapies, such as chemotherapy, radiotherapy, and immunotherapy (1, 2). The single-agent gemcitabine is known to be an acceptable standard in advanced and metastatic pancreatic cancer, but the benefit is small. Even the addition of other chemotherapeutics or monoclonal antibodies with gemcitabine has not resulted in a meaningful improvement in survival of pancreatic cancer patients (2).

Signal transducers and activators of transcription (*STAT*) belong to a family of transcription factors that relay cytokine receptor-generated signals into the nucleus. The phosphorylation of Janus kinases (*JAK*) via the activation of cytokine receptors creates receptor docking sites for recruitment of cytoplasmic *STAT* proteins. Additionally, activation of *STAT* signaling is also modulated by several growth factors such as epidermal growth factor, hepatocyte growth factor, and platelet-derived growth factor. The activated *STATs* translocate to the nucleus in a dimer form, bind to specific DNA response elements, and finally modulate cell growth and differentiation (3, 4). Recent studies showed aberrant signaling of the *JAK/STAT* pathway in pancreatic cancer (4, 5), which therefore may provide a novel therapeutic strategy for this type of aggressive cancer.

Biochemists and biologists have been investigating a variety of purified compounds from herbs as possible sources of new anticancer drugs. Naturally occurring cucurbitacins constitute a group of oxygenated triterpenes, which are characterized by the tetracyclic cucurbitane nucleus skeleton and present in many plants as β -glucosides. Cucurbitacin B, a member of the cucurbitacins, is extracted from *Trichosanthes kirilowii Maximowicz* (Cucurbitaceae family), a plant that has long been used in oriental medicine for its anti-inflammatory, antidiabetic, and abortifacient effects. This active ingredient has antiproliferative effects on several types of malignancies and is known to be an dual inhibitor of the activation of both *JAK2* and *STAT3* in some malignancies (3). Recently, we were able to show that cucurbitacin B has antiproliferative activity against human breast cancer, glioblastoma multiforme, and myeloid leukemia cells (6–8).

In the present study, we show that cucurbitacin B markedly inhibits the proliferation of seven human pancreatic cancer cell lines. The growth-inhibitory effects of this compound were associated with a significant G₂-M-phase arrest and increase in apoptosis by inhibition of *JAK2*, *STAT3*, and *STAT5* and subsequent activation of the *caspase* cascade. Moreover, the compound significantly potentiated the antiproliferative effects of gemcitabine on pancreatic cancer cells. We further confirmed our *in vitro*

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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observations by showing profound antitumor activity of cucurbitacin B in a murine xenograft model with no apparent toxicity to the animals.

Materials and Methods

Cell culture and compounds. Human pancreatic cancer cell lines were obtained from the American Type Culture Collection. Monolayer cultures of MiaPaCa-2, PL45, and Panc-1 were maintained in DMEM (Life Technologies), SU86.86 and AsPC-1 were maintained in RPMI 1640, and Panc-03.27 and Panc-10.05 were maintained in RPMI 1640 supplemented with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, and 10 units/mL human insulin. Heat-inactivated fetal bovine serum (10%, v/v; Gemini Bio-Products) was added to all cell cultures, and they were maintained at 37°C in a humidified chamber of 95% air and 5% CO₂. Pancreatic adenocarcinoma cells growing as a monolayer were detached from the flask surface using 2.5% trypsin-EDTA solution. Cell counts were determined using a hemocytometer (Allegiance Healthcare), and only cells in the logarithmic phase of growth were used for all studies.

In this study, we used highly purified cucurbitacin B (CKBP002; molecular weight 558.7; C₃₂H₄₆O₈; Fig. 1) synthesized by CK Life Sciences Int'l. CKBP002 consists of cucurbitacin B with >99% purity as confirmed by high-performance liquid chromatography (data provided on request by CK Life Sciences Int'l). The drug was dissolved in DMSO at a stock concentration of 10⁻² mol/L and stored at -20°C.

Gemcitabine (Gemzar; Eli Lilly) was stored at 4°C and dissolved in sterile PBS on the day of use.

Measurement of cellular proliferation and colony count. For measurement of proliferation, the seven human pancreatic cancer cell lines were placed into 96-well plates at 10⁴ cells per well (100 μL) and treated with either diluent control (DMSO) or various concentrations of cucurbitacin B (10⁻⁹-10⁻⁶ mol/L). The cells were incubated at 37°C for 96 h, a time point selected as it was previously determined to be optimal for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (9). MTT (10 μL; 5 mg/mL; Sigma) dissolved in PBS was added to each well followed by 100 μL of 20% SDS after 4 h. The absorbance of the product was measured with an ELISA reader at 540 nm after 16 more hours. The obtained values of 50% effective dose were expressed as the concentrations that corresponded to a reduction of cellular growth by 50% when compared with values of the diluent control cells.

For pulse-exposure experiments, all seven human pancreatic cancer cell lines were exposed to cucurbitacin B (10⁻⁷ mol/L) for either 2, 9, or 20 h, washed with culture medium, and cultured again in cucurbitacin B-free medium. After an additional 1, 2, and 3 days, MTT assay was done as described above. To examine if cucurbitacin B treatment influences the ploidy in pancreatic cancer cell lines, cells were drug-treated for 9 h (10⁻⁷ mol/L) and incubated for an additional 24 h in drug-free medium.

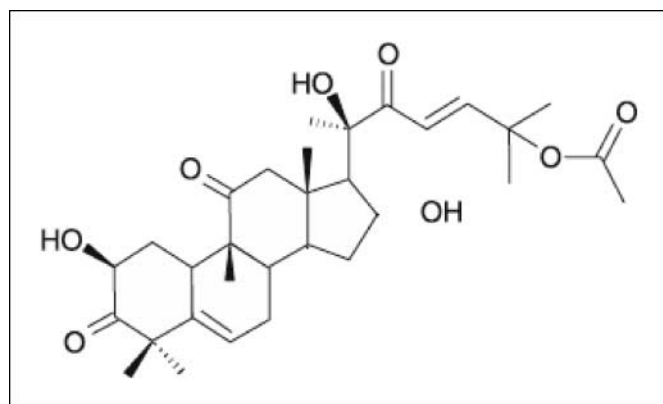


Figure 1. Chemical structure of the triterpenoid cucurbitacin B.

After being trypsinized and washed twice with ice-cold PBS, the cells were fixed in 70% methanol overnight. The fixed samples were treated with RNase A, stained with propidium iodide, and analyzed by flow cytometry (FACScan; Becton Dickinson) with the ModFitLT V2.0 software (Verity).

For clonogenic assays, three pancreatic cancer cell lines (MiaPaCa-2, PL45, and Panc-1) were plated into 24-well plates using a two-layer soft agar system with a total of 3 × 10³ cells per well in a volume of 400 μL/well as described previously (10). After 10 days of incubation with different concentrations of cucurbitacin B (10⁻⁹-10⁻⁷ mol/L), the colonies were counted.

Cell cycle analysis and measurement of apoptosis. Two pancreatic cancer cell lines (10⁵ cells; Panc-1 and MiaPaCa-2) were analyzed for alterations in their cell cycle after treating the cells for 24, 48, and 72 h with cucurbitacin B (5 × 10⁻⁸ and 10⁻⁷ mol/L). After the cells were fixed in 70% methanol, the samples were treated with RNase A and exposed to propidium iodide for analysis by flow cytometry.

We used the Annexin V assay (Annexin V-FITC Apoptosis Detection Kit; BD Pharmingen) for the measurement of cellular apoptosis. The pancreatic cancer cell lines Panc-1, MiaPaCa-2, and PL45 (10⁵ cells) were cultured with cucurbitacin B (2 × 10⁻⁷ mol/L) for either 24, 48, 72, or 96 h. Annexin V-FITC and propidium iodide labeling followed, which was done according to the manufacturer's instructions. Percentage of apoptosis in both diluent control and cucurbitacin B-treated cells was analyzed by flow cytometry.

Western blotting. Panc-1 pancreatic cancer cells with a known *p53* mutation were harvested for Western blotting, and proteins were extracted with radioimmunoprecipitation assay buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris-HCl (pH 7.5)] containing protease inhibitor cocktail (Roche Molecular Biochemicals). Protein lysates (50 μg) were boiled in Laemmli sample buffer (Bio-Rad Laboratories), resolved by electrophoresis on 4% to 15% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Membranes were probed with antibodies from Cell Signaling Technology as well as Santa Cruz Biotechnology and developed using the enhanced chemiluminescence kit (Pierce), whereas GAPDH was used as a control. All antibodies that were used in this study can be found in Supplementary Table S1.

Murine xenograft model and tumor treatment. The *in vivo* behavior of pancreatic cancers was investigated in a murine xenograft model. All animal experiments were in accordance with the guidelines of Cedars-Sinai Research Institute and the NIH. Five-week-old female *nu/nu* athymic mice (weight 20-22 g; specific pathogen-free) from Harlan Sprague-Dawley were maintained in pathogen-free conditions and fed irradiated chow. Mice (5 per cohort) were randomly assigned to either treatment group (mean weight 21.1 g) or control group (mean weight 20.9 g). Panc-1 cells (10⁷) in 0.2 mL Matrigel (Basement Membrane Matrix, High Concentration; BD Biosciences) were injected subcutaneously in both flanks of the nude mice. Because each mouse had two tumors, every group consisted of 10 tumors. Treatment was started the day after cell implantation with either 1 mg/kg body weight intraperitoneal injections of cucurbitacin B three times a week (treatment group) or diluent control (DMSO) alone (control group). We showed previously that this concentration of the drug and frequency of administration were safe for mice (6). Cucurbitacin B was dissolved in 5% DMSO and administered to the mice in a volume of 0.1 mL. Tumors were measured with Vernier calipers every 3 days and volume was calculated as described previously using the following formula: (length × width × depth) × 0.5236 (11). Each mouse was weighed twice per week. After 48 days of treatment, the experiment was halted due to excessive tumor volume in the control group. We weighed the dissected tumors, fixed them in 10% neutral-buffered formalin, and embed these tumors them in paraffin wax. Inhibition rate of tumor growth was calculated using the following formula: tumor growth inhibition rate (%) = (1 - M_T / M_C) × 100, where M_T and M_C are the mean normalized tumor masses of treatment and control groups, respectively (12). Metastatic disease was determined macroscopically at autopsy in all thoracic, abdominal, retroperitoneal, and pelvic organs followed by histologic examination. At sacrifice, tumors were harvested from mice, and a portion of each tumor was snap-frozen in liquid nitrogen and stored at -80°C until analysis. Tumor tissue homogenates were prepared in SDS

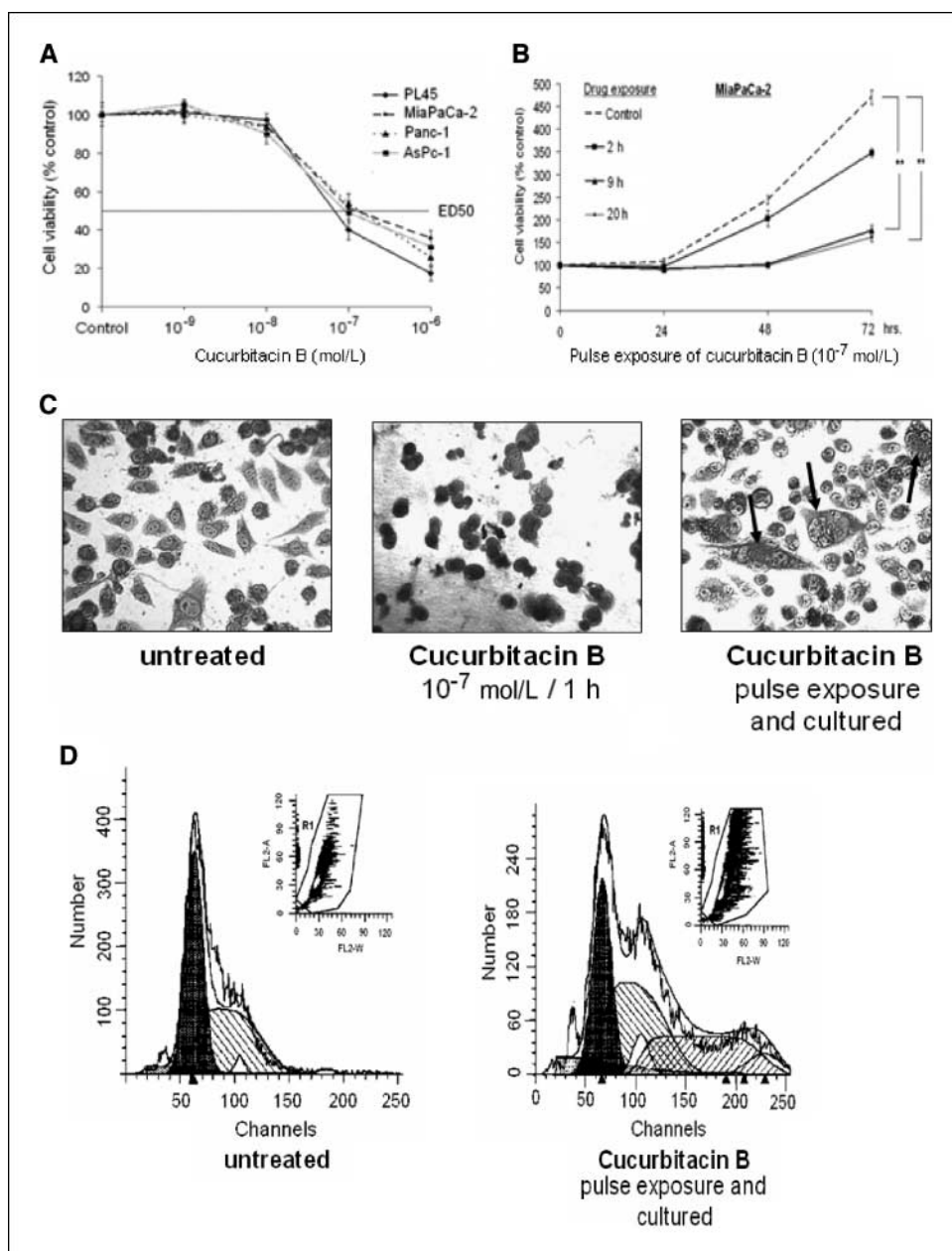


Figure 2. Cucurbitacin B inhibited proliferation of pancreatic cancer cells and caused multinucleation. **A**, proliferation of pancreatic cancer cell lines exposed to cucurbitacin B (10^{-9} – 10^{-6} mol/L) was tested via MTT assay. **B**, MiaPaCa-2 cell line was pulse exposed to 10^{-7} mol/L cucurbitacin B (2, 9, and 20 h) and recultured in absence of cucurbitacin B (24, 48, and 72 h). **, $P < 0.01$. **C**, MiaPaCa-2 cell line (untreated; left) exposed for 1 h to 10^{-7} mol/L cucurbitacin B (middle), pulse-exposed for 9 h with 10^{-7} mol/L cucurbitacin B, and recultured in normal medium for an additional 24 h (right). Arrows, multinucleation. Wright staining, $\times 40$. **D**, analysis for tetraploidy versus diploidy by flow cytometry (ModFitLT V2.0 software) after 9 h exposure of MiaPaCa-2 to 10^{-7} mol/L cucurbitacin B followed by 24 h of culture in drug-free medium; cells were gated using FL2-W versus FL2-A bivariate graphs. Black triangles, detection of tetraploidy.

lysis buffer of 50 mmol/L Tris-HCl (pH 7.4), 2% SDS, and protease inhibitor mixture as described previously (13).

Statistical analysis. All *in vitro* experiments were repeated at least three times to confirm the results, and data were expressed as the mean \pm SD. Statistical significance was determined with Student's *t* test (two-tailed) when comparing two groups of data set. *P* values < 0.05 were considered statistically significant. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition (*, $P < 0.05$; **, $P < 0.01$).

Drug synergy was determined by the isobologram and combination index (CI) methods (CalcuSyn software, version 1.1.1 1996; Biosoft) derived from the median-effect principle of Chou and Talalay (14, 15). The isobologram method is a graphical representation of the pharmacologic interaction and is formed by selecting a desired fractional cell kill and plotting the required individual drug doses on their respective *x* and *y* axes. A straight line is then drawn to connect the points. The observed dose combination of the two agents that achieved that particular fractional cell kill is then plotted on the

isobologram. Combination data points that fall on the line represent an additive drug-drug interaction, whereas data points that fall below or above the line represent either synergism or antagonism, respectively. The CI method is a mathematical and quantitative representation of a two-drug pharmacologic interaction. Using data from the growth-inhibitory experiments and computerized software, CI values are generated over a range of *F*_a levels from 0.05 to 0.90 (5–90% growth inhibition). A CI of 1 indicates an additive effect between two agents, whereas a CI < 1 or CI > 1 indicates synergism or antagonism, respectively.

Results

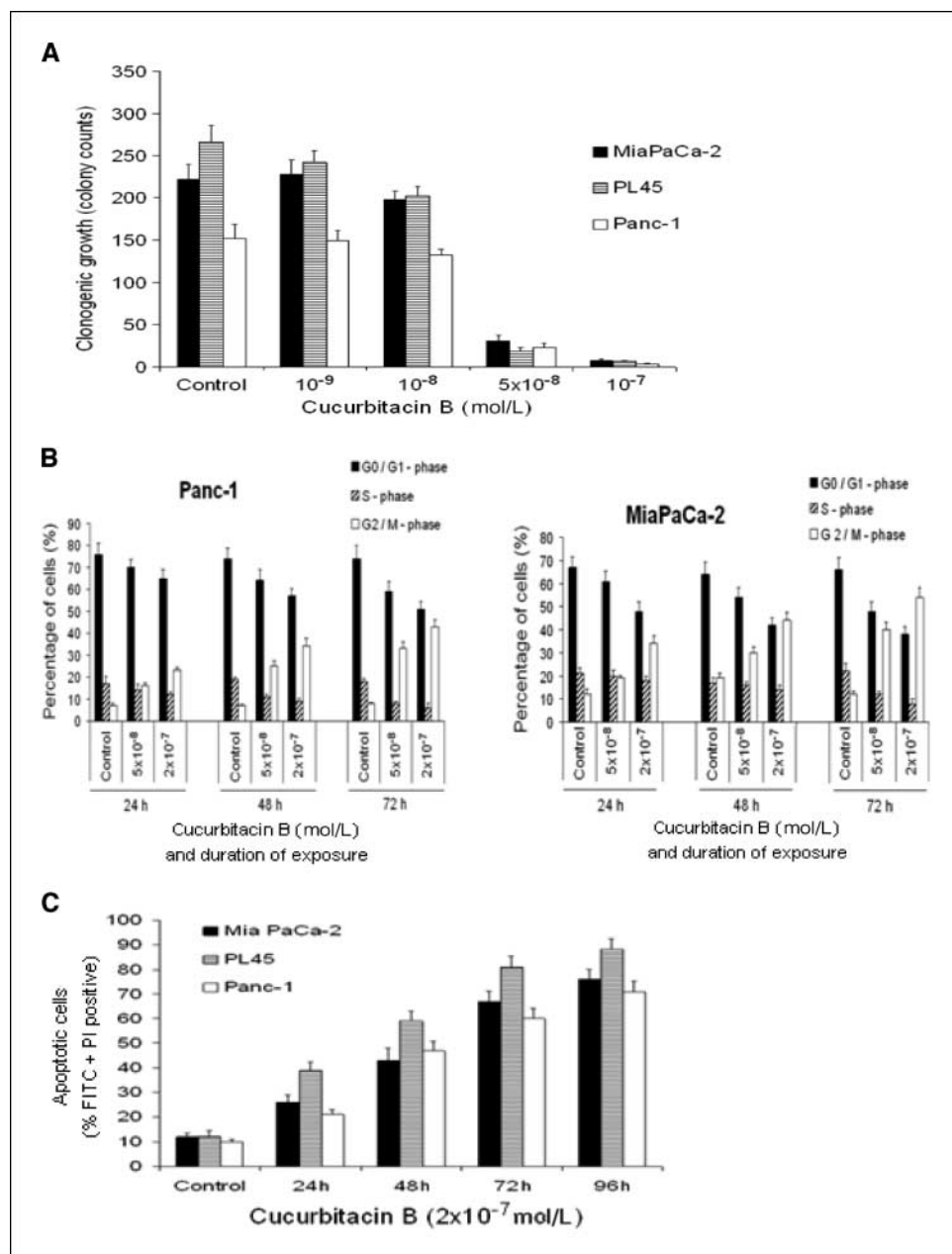
Cucurbitacin B inhibits proliferation of pancreatic cancer cells and causes multinucleation. We tested the ability of cucurbitacin B to inhibit the proliferation of a panel of seven pancreatic cancer cell lines via the MTT assay: MiaPaCa-2, PL45,

Panc-1, and AsPC-1 (Fig. 2A) as well as SU86.86, Panc-03.27, and Panc-10.05 (data not shown). Each had a fairly similar sensitivity to the compound with an inhibition of 50% growth at 10^{-7} mol/L. We also examined the antiproliferative activity of the seven malignant cell lines when pulse-exposed to cucurbitacin B (10^{-7} mol/L) for either 2, 9, or 20 h, washed, and grown in liquid culture in the absence of cucurbitacin B for an additional 1, 2, or 3 days. A significant decrease in proliferation of the pancreatic cancer cells occurred after exposure for either 9 or 20 h to the drug compared with the diluent-exposed control cells (Fig. 2B; representative data shown for MiaPaCa-2). The pancreatic cancer cell lines underwent rapid morphologic changes within <1 h after exposure to cucurbitacin B, rounding up and losing their pseudopodia (Fig. 2C). Reexamining the cells after growing in normal culture medium, the cells reverted to their regular morphology within

24 h after pulse exposure, suggesting that the alterations mediated by 1 h of exposure to cucurbitacin B were reversible. Interestingly, the pulse-exposure treatment for either 9 or 20 h with 10^{-7} mol/L cucurbitacin B caused prominent multinucleation of the cells after they were washed and recultured in normal medium for an additional 24 h (Fig. 2C). After 9 h of exposure of MiaPaCa-2 and Panc-1 cell lines to 10^{-7} mol/L cucurbitacin B followed by 24 h of culture in drug-free medium, a mean of $38 \pm 7\%$ cells developed tetraploidy ($62 \pm 7\%$ diploidy) compared with $4 \pm 2\%$ in untreated cells ($96 \pm 2\%$ diploidy; Fig. 2D).

Cucurbitacin B inhibits clonogenic growth, produces cell cycle arrest, and increases apoptosis of pancreatic cancer cells. Clonogenic assay relies on individual colony-forming cells to proliferate actively in soft gel culture to form a colony of cells. When pancreatic cancer cell lines (MiaPaCa-2, PL45, and Panc-1)

Figure 3. Cucurbitacin B caused inhibition of clonogenic growth, cell cycle arrest, and increased level of apoptosis of pancreatic cancer cells. *A*, pancreatic cancer cell lines (MiaPaCa-2, PL45, and Panc-1) were cultured in soft agar for 10 days either with or without cucurbitacin B (10^{-9} – 10^{-7} mol/L), and colonies were counted. *B*, cell cycle distribution of Panc-1 and MiaPaCa-2 was measured by flow cytometry after exposure to cucurbitacin B (5×10^{-8} and 2×10^{-7} mol/L) for 24, 48, and 72 h. *C*, apoptosis was measured by Annexin V-FITC and propidium iodide labeling of 10^5 pancreatic cancer cells (MiaPaCa-2, PL45, and Panc-1) after treatment with 2×10^{-7} mol/L cucurbitacin B for 24, 48, 72, and 96 h.



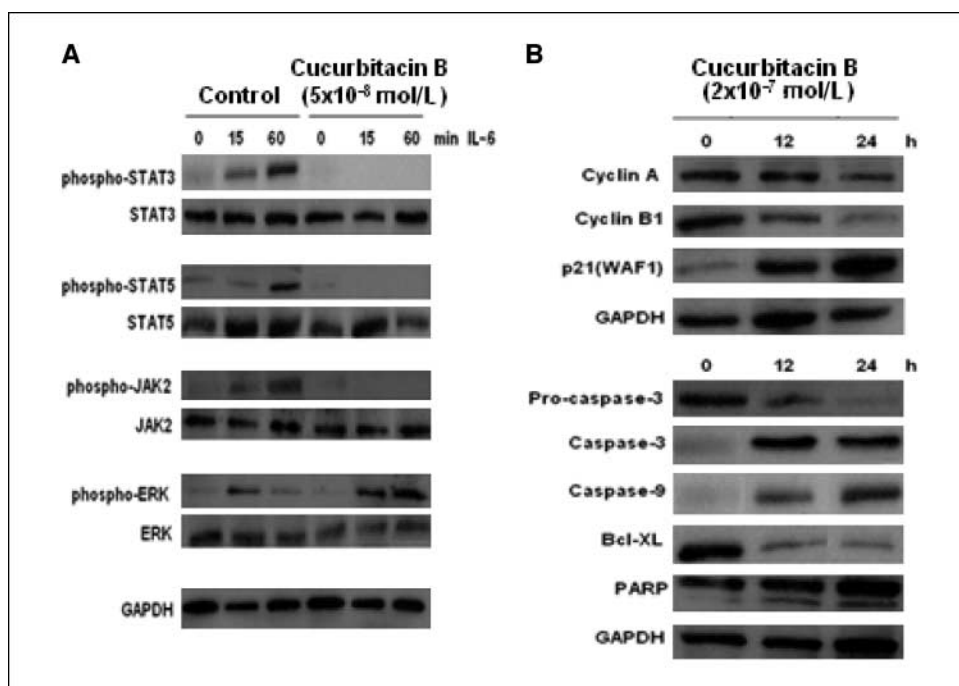


Figure 4. Cucurbitacin B profoundly inhibited JAK/STAT pathway with activation of the caspase cascade. Western blot analysis of Panc-1 cells treated with cucurbitacin B. A, cells were incubated either with or without the drug (5×10^{-8} mol/L) overnight in the absence of serum. Interleukin-6 (100 ng/mL) was then added to control and drug-treated wells for the indicated times. B, cells were exposed to 2×10^{-7} mol/L cucurbitacin B for 24 h.

were cultured in soft agar either with or without cucurbitacin B, clonal growth was inhibited significantly by the drug in a dose-dependent fashion compared with the control cells. Cucurbitacin B at 5×10^{-8} mol/L inhibited >80% of the clonogenic pancreatic cancer cells (Fig. 3A).

The affect of cucurbitacin B on the cell cycle was studied by flow cytometry using MiaPaCa-2 and Panc-1 pancreatic cancer cell lines. The compound caused a time- and dose-dependent growth arrest in the G_2 -M phase of the cell cycle (Fig. 3B; representative data for MiaPaCa-2 shown). After 72 h of drug exposure (2×10^{-7} mol/L), the percentage of cells in the G_2 -M phase increased up to 5-fold in the treated cell lines compared with the controls.

Further, the ability of cucurbitacin B (10^{-7} mol/L) to induce apoptosis was measured by Annexin V-FITC and propidium iodide labeling of pancreatic cancer cells. A total of $76 \pm 4\%$ (MiaPaCa-2), $85 \pm 4.5\%$ (PL45), and $71 \pm 4\%$ (Panc-1), respectively, of the pancreatic cancer cells were apoptotic after 96 h of drug exposure, whereas only $12 \pm 2\%$ (MiaPaCa-2), $12.8 \pm 2.3\%$ (PL45), and $10 \pm 1\%$ (Panc-1), respectively, of diluent treated control cells had undergone apoptosis (Fig. 3C).

Cucurbitacin B profoundly inhibits JAK/STAT pathway with activation of caspase cascade. The JAK/STAT pathway plays an important role in cell growth, proliferation, and survival, and it has been implicated in many human cancers (16, 17). We tested the ability of cucurbitacin B to modulate the expression of these proteins. Serum-starved Panc-1 cells with mutant *p53* expressed high levels of activated JAK2, STAT3, and STAT5 in response to interleukin-6 treatment (Fig. 4A). In contrast, the activation of JAK2, STAT3, and STAT5 was markedly inhibited by 4 h treatment with cucurbitacin B (5×10^{-8} mol/L). The RAS/RAF/ERK and PI3K/AKT signaling pathways were not down-regulated by the compound. Activation of AKT and JNK was not affected (data not shown), whereas phosphorylated ERK was up-regulated by drug treatment (Fig. 4A). We further examined the expression of proteins involved in cell cycle regulation and apoptotic pathways, which are known to be connected to the JAK/STAT pathway. Cyclin

kinase inhibitor *p21^{WAF1}*, which is associated with cell cycle arrest and apoptosis (18), was increased in a time-dependent manner, and the cell cycle regulators *cyclin A* and *cyclin B1* decreased after 24 h exposure to cucurbitacin B (Fig. 4B). The drug also decreased the protein level of the antiapoptotic *Bcl-XL* protein and increased the proapoptotic *caspase-3* and *caspase-9* enzyme levels associated with PARP cleavage over 24 h. The protein expression of the cell cycle inhibitor *p27 (CDKN1B)* as well as *cyclin D1* were not altered by the drug treatment (data not shown).

Cucurbitacin B synergistically potentiates the antiproliferative effects of gemcitabine. The growth of MiaPaCa-2 and Panc-1 cells treated with cucurbitacin B (10^{-8} , 5×10^{-8} , and 10^{-7} mol/L), gemcitabine (10^{-9} and 10^{-8} mol/L), or a combination of both was determined by MTT assay. The dose in this study was chosen based on a preliminary dose-escalation study (data not shown). We observed no significant combinatory effects in growth inhibition when using either cucurbitacin B 10^{-8} or 5×10^{-8} mol/L together with gemcitabine 10^{-9} mol/L (treatments 1 and 2, respectively; Fig. 5). In contrast, a significant reduction in growth was observed in both cell lines treated in a combination of cucurbitacin B 10^{-7} mol/L with gemcitabine 10^{-9} mol/L (treatment 3) as well as in combination of cucurbitacin B 10^{-8} , 5×10^{-8} , or 10^{-7} mol/L together with 10^{-8} mol/L gemcitabine (treatments 4-6, respectively; $P < 0.01$; Fig. 5). With these latter combinations, the CI was <0.9 in both cell lines, which means that the two drugs have a synergistic activity.

Cucurbitacin B markedly decreases growth of Panc-1 human pancreatic tumor xenografts in vivo. We also determined whether cucurbitacin B administration can suppress the growth of human pancreatic tumor xenografts *in vivo*. Panc-1 pancreatic tumor cells were subcutaneously implanted in athymic nude mice, and the experimental mice were given 1 mg/kg cucurbitacin B intraperitoneally 3 days a week. The experiment ended on day 48 due to excessive tumor size in the diluent-treated control group. Growth of the tumors was significantly inhibited in the mice treated with cucurbitacin B compared with the growth of

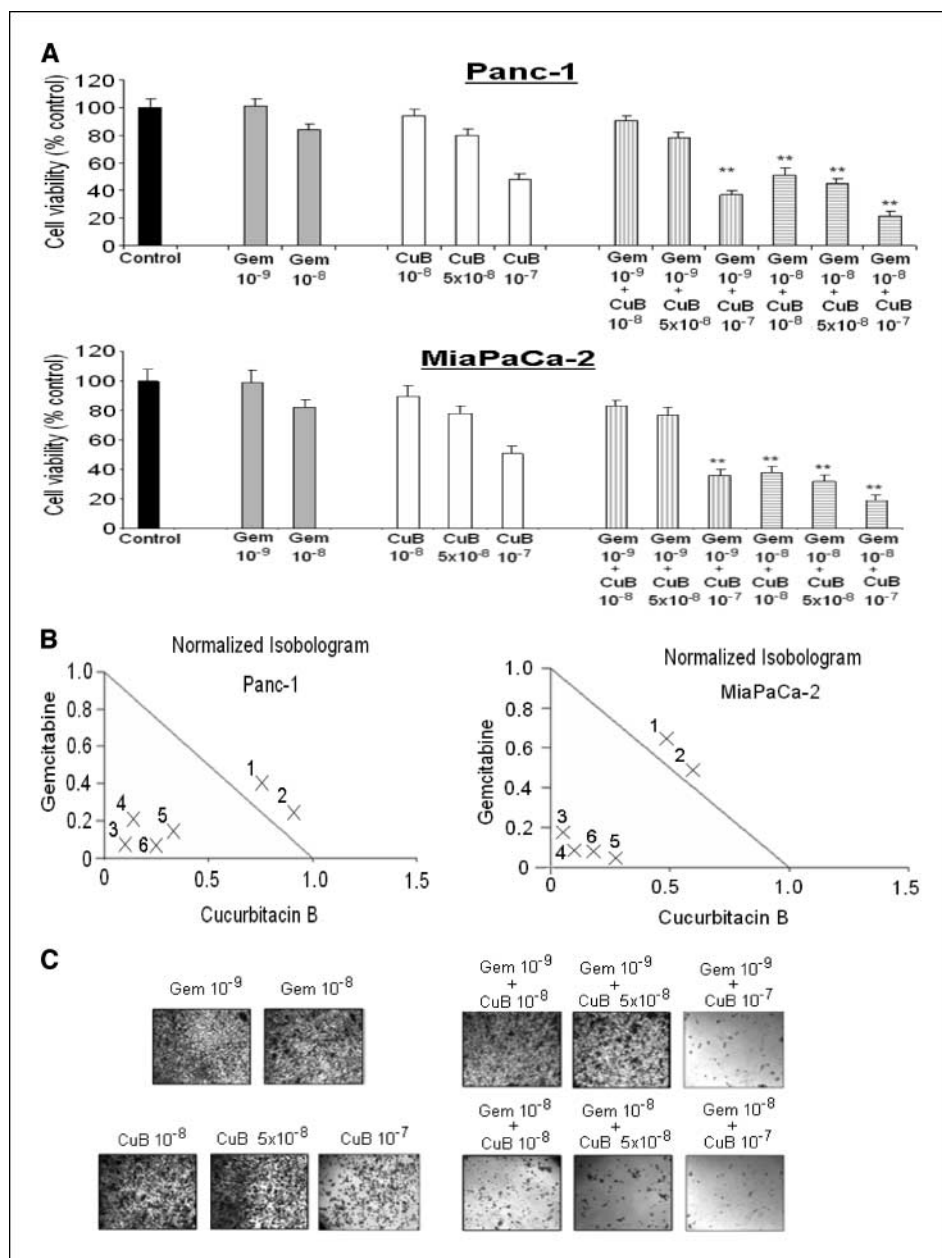
tumors in control mice (Fig. 6A). At the conclusion of the study, the mean tumor volume in the diluent treated control mice was $1.8 \pm 0.24 \text{ cm}^3$ compared with $0.51 \pm 0.14 \text{ cm}^3$ in the cucurbitacin B-treated mice (69.2% decrease; $P < 0.01$; Fig. 6B). The overall tumor weight of the control group was $19.4 \pm 2.12 \text{ g}$, whereas it was only $3.96 \pm 0.61 \text{ g}$ for the mice that received cucurbitacin B treatment ($P < 0.01$; Fig. 6C). Furthermore, no metastases were found in the cucurbitacin B-treated group, whereas the control cohort showed additional metastatic spread in the axillary lymph nodes, mediastinum, liver, and peritoneum, which was confirmed by histologic examination of suspected lesions. Treatment with cucurbitacin B was without apparent ill consequences for the mice such as altered weight, signs of discomfort, or impaired movement (data not shown). Concordantly with our *in vitro* results, tumors of mice treated with cucurbitacin B had lower levels of activated *STAT3*, *JAK2*, and the antiapoptotic factor *Bcl-XL* and higher

expression of caspase-3 and caspase-9 compared with the tumors from the control mice (Fig. 6D).

Discussion

In 1997, a randomized phase III study of gemcitabine versus 5-fluorouracil showed a statistically significant clinical benefit of gemcitabine compared with 5-fluorouracil (19). The 1-year survival rate of patients with pancreatic cancer treated with gemcitabine was 18% versus 2% with 5-fluorouracil. Since that time, the nucleoside analogue gemcitabine has become standard chemotherapy for locally advanced and metastatic pancreatic cancer. Limited progress has been achieved despite intense research using combinatorial treatments with chemotherapy agents, often along with the use of biological agents targeting activated molecular pathways involved in pancreatic cancer. For instance, erlotinib, an

Figure 5. Cucurbitacin B synergistically potentiated the antiproliferative effects of gemcitabine. **A**, proliferation of Panc-1 and MiaPaCa-2 cells treated with either cucurbitacin B (10^{-8} , 5×10^{-8} , and 10^{-7} mol/L), gemcitabine (10^{-9} and 10^{-8} mol/L), or a combination of both was determined by MTT assay. The dose in this study was chosen based on a preliminary dose escalation study. *CuB*, cucurbitacin B; *Gem*, gemcitabine. **B**, drug synergy was determined by the isobologram calculation (CalcuSyn software, version 1.1.1). Combinations used: 1, gemcitabine 10^{-9} mol/L and cucurbitacin B 10^{-8} mol/L; 2, gemcitabine 10^{-9} mol/L and cucurbitacin B 5×10^{-8} mol/L; 3, gemcitabine 10^{-9} mol/L and cucurbitacin B 10^{-7} mol/L; 4, gemcitabine 10^{-8} and cucurbitacin B 10^{-8} mol/L; 5, gemcitabine 10^{-8} and cucurbitacin B 5×10^{-8} mol/L; 6, gemcitabine 10^{-8} and cucurbitacin B 10^{-7} mol/L. **, $P < 0.01$. **C**, MiaPaCa-2 cells were stained with MTT after treatment with different concentrations (mol/L) of gemcitabine and/or cucurbitacin B. Magnification, $\times 20$.



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oral tyrosine kinase inhibitor that selectively inhibits the epidermal growth factor receptor, was recently tested in combination with gemcitabine (20). However, due to a small improvement in median survival and the resultant toxicity, the clinical benefit of this combination remains unclear. Moreover, agents inhibiting vascular endothelial growth factor pathways have been largely disappointing as primary therapy. For example, bevacizumab in combination with gemcitabine in a randomized phase III clinical trial as well as sorafenib in a small pilot study have both failed to show superiority over gemcitabine monotherapy (21, 22). Patients with refractory advanced pancreatic cancer have had a benefit from salvage chemotherapy with 5-fluorouracil/oxaliplatin-based regimens (23, 24).

However, many new insights in the molecular alterations and their prognostic significance concerning pancreatic cancer have been made over the last years. Recently, studies provided evidence that the *JAK/STAT3* pathway, which is well known to play a major role in many types of cancers, may enhance proliferation of pancreatic cancer. This pathway is often constitutively activated in subsets of human pancreatic cancer tissues and cell lines (4, 5). Moreover, specific inhibitors of *JAK2* and/or *STAT3* decrease growth of pancreatic cancer cell lines. *STAT5* is also known to be active in a variety of malignancies such as breast and prostate cancer, leukemias, and myeloproliferative disorders (25, 26).

In this study, we provide strong evidence that cucurbitacin B has a marked antiproliferative effect on pancreatic cancer at least in part by the inhibition of the *JAK/STAT* signaling pathway. This compound inhibited the activation of *JAK2*, *STAT3*, and *STAT5*.

Downstream targets of the *JAK/STAT* pathway, which are responsible for generating pro-proliferative signals via cell cycle modulation and up-regulate antiapoptotic proteins, were also affected. Cyclin-dependent kinase inhibitor *p21^{WAF1}*, which acts as a negative cell cycle regulator, was up-regulated; and *cyclin A* and *cyclin B1* were down-regulated by cucurbitacin B treatment. This resulted in a dose- and time-dependent G₂-M cell cycle arrest in drug-treated pancreatic cancer cells. In general, about half of human cancers have p53 mutations resulting in the inability of this protein to induce expression of p21^{WAF1} (27, 28). However, p21^{WAF1} not only mediates growth arrest and apoptosis in response to DNA damage but may also be responsible in a p53-independent way for the antiproliferative effects of certain growth inhibitory stimuli, such as *transforming growth factor-β* (29), mimosine (30), okadaic acid, 12-*O*-tetradecanoylphorbol-13-acetate (31), and several differentiating agents (32). Because Panc-1 pancreatic cancer cells have p53 mutations, cucurbitacin B is able to up-regulate expression of p21^{WAF1} independent of p53. This is relevant because p53 mutations have been found in 40% to 76% of pancreatic cancer tissues (28).

Pulse-exposure experiments showed that only 9 h exposure to 10⁻⁷ mol/L cucurbitacin B inhibited growth of up to 81% pancreatic cancer cells. Additionally, these cells showed prominent multinucleation with high levels of tetraploidy, one important sign of failed mitosis, even after cells were reexposed to drug-free medium. Interestingly, after a very brief exposure (<1 h) to cucurbitacin B, the pancreatic cancer cells became round and refractile. These morphologic changes are similar to changes we

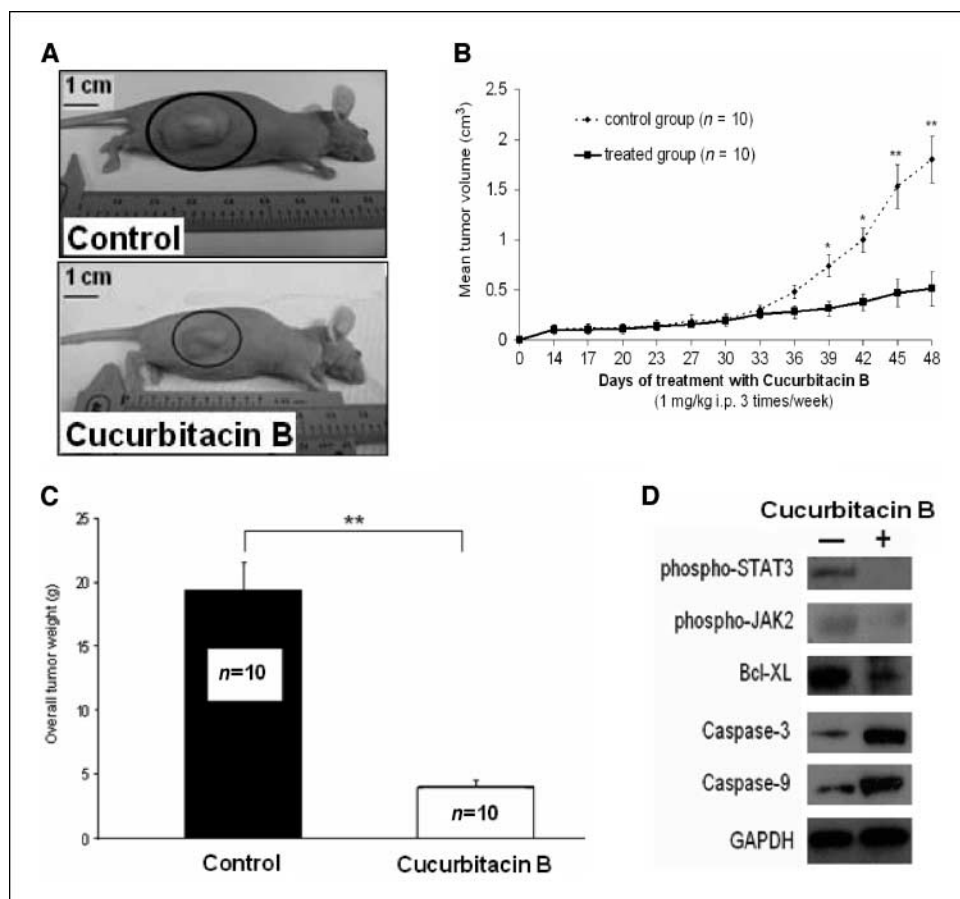


Figure 6. Cucurbitacin B inhibited the growth of Panc-1 human pancreatic tumor xenografts *in vivo*. **A**, Panc-1 cells (1×10^7) were subcutaneously implanted into both flanks of 10 athymic *nu/nu* mice. Cucurbitacin B (1 mg/kg three times a week; 5 mice) or vehicle (5 mice) were given intraperitoneally for 48 days. **B**, after 48 days of therapy, 10 tumors from both treated and untreated group were dissected, and their volumes were measured. Bars, SD. *, $P < 0.05$; **, $P < 0.01$. **C**, overall weight of the dissected tumors. Columns, mean overall tumor weight; bars, SD. **, $P < 0.01$. **D**, tumor lysates were used for Western blotting to analyze the *in vivo* effect of cucurbitacin B on the *JAK/STAT* pathway and its downstream targets.

noted in studies with cucurbitacin B on human breast cancer and glioblastoma cell lines (6, 7). In those two studies, we showed by confocal microscopy that the drug disrupted the cytoskeletal architecture including F-actin, whereas the spindle microtubules were still functional.

Bcl-XL plays a vital role in pancreatic cancer chemoresistance, and the increased expression of this antiapoptotic protein is associated with poor survival in this type of cancer (33, 34). Cucurbitacin B was markedly able to decrease *Bcl-XL* expression in pancreatic cancer cells and activate the *caspase* cascade as another important downstream target of *JAK/STAT* signaling. The activity of *caspase* family members 3 and 9 were up-regulated by cucurbitacin B treatment with subsequent cleavage of *PARP* protein. This activation was associated with increased levels of apoptosis in the pancreatic cancer cell lines. The *RAS/RAF/ERK* and *PI3K/AKT* pathways were not inhibited by cucurbitacin B, suggesting that these signaling pathways are not important for growth inhibition or apoptosis of pancreatic cancer cells by the drug.

Pancreatic cancer is poorly treated by conventional therapies including gemcitabine, whereas aberrant activation of the *STAT* pathway is able to confer resistance to radiation and chemotherapies (35, 36). The ability of cucurbitacin B to potentiate gemcitabine is notable. Strikingly, the drug at 10^{-8} mol/L synergistically augmented the antiproliferative effect of gemcitabine. However, further *in vivo* studies are warranted to confirm these findings.

The cucurbitacins as unpurified molecules have been used for centuries as folk medicine in countries such as China and India without any clear toxicity (37). They have been used because of their anti-inflammatory analgesic effects. We showed that administration of purified cucurbitacin B to athymic nude mice with human pancreatic cancer xenografts significantly retarded tumor growth. In agreement with our *in vitro* observations, tumors from these mice had decreased expression of activated *JAK2* and *STAT3* compared with controls. The enhanced expression of the antiapoptotic gene *Bcl-XL* in pancreatic cancer and its association

with shorter patient survival suggests that this protein may enhance the viability of pancreatic cancer cells *in vivo*. Interestingly, we found that cucurbitacin B inhibited the *in vivo* expression of *Bcl-XL* with increased activity of the proapoptotic caspases. Regarding *in vivo* toxicity, the cucurbitacin B-treated mice tolerated the therapy with no signs of toxicity including no significant change in body weight. This is similar to our previous study of administration of cucurbitacin B to mice having human breast cancer xenografts (6). These mice also received intraperitoneal injections of 1 mg/kg cucurbitacin B three times a week with no apparent side effects.

In the present study, we show for the first time that cucurbitacin B has profound antipancreatic cancer activity *in vitro* and *in vivo*. The drug induces cell cycle arrest and apoptosis in pancreatic cancer cell lines by inhibiting the *JAK/STAT* pathway, increasing the expression of *p21^{WAF1}* independently of *p53* activity, and inducing the *caspase* cascade. The drug also potentiates the proliferative activity of the nucleoside analogue gemcitabine. Our studies provide a rationale for the development of cucurbitacin B as a therapeutic agent against human pancreatic cancer.

Disclosure of Potential Conflicts of Interest

M. Toh and W.D. Xie: Ownership interest, CK Life Science, Int'l. H.P. Koeffler: Commercial research grant, CK Life Science, Int'l. The other authors disclosed no potential conflicts of interest.

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