

Suppression of pancreatic tumor growth by combination chemotherapy with sulindac and LC-1 is associated with cyclin D1 inhibition *in vivo*

Michele T. Yip-Schneider,^{1,6} Huangbing Wu,¹ Matthew Ralstin,¹ Constantin Yiannoutsos,⁴ Peter A. Crooks,⁸ Sundar Neelakantan,⁸ Stephen Noble,¹ Harikrishna Nakshatri,^{1,2,5,6} Christopher J. Sweeney,^{3,6} and C. Max Schmidt^{1,2,5,6,7}

Departments of ¹Surgery, ²Biochemistry/Molecular Biology, ³Hematology/Oncology, and ⁴Biostatistics and ⁵Walther Oncology Center, Indiana University School of Medicine; ⁶Indiana University Cancer Center; ⁷Richard L. Roudebush VA Medical Center, Indianapolis, Indiana; and ⁸College of Pharmacy, University of Kentucky, Lexington, Kentucky

Abstract

The design of novel targeted or combination therapies may improve treatment options for pancreatic cancer. Two targets of recent interest are nuclear factor- κ B (NF- κ B) and cyclooxygenase (COX), known to be activated or overexpressed, respectively, in pancreatic cancer. We have previously shown that parthenolide, a proapoptotic drug associated with NF- κ B inhibition, enhanced the growth suppression of pancreatic cancer cells by the COX inhibitor sulindac *in vitro*. In the present study, a bioavailable analogue of parthenolide, LC-1, and sulindac were evaluated *in vivo* using a xenograft model of human pancreatic cancer. Treatment groups included placebo, low-dose/high-dose LC-1 (20 and 40 mg/kg), low-dose/high-dose sulindac (20 and 60 mg/kg), and low-dose combination LC-1/sulindac (20 mg/kg each). In MiaPaCa-2 xenografts, tumor growth was inhibited by either high-dose sulindac or LC-1. In BxPC-3 xenografts, tumor size was significantly reduced by treatment with the low-dose LC-1/sulindac combination or high-dose sulindac alone ($P < 0.05$). Immunohistochemistry of BxPC-3 tumors

revealed a significant decrease in Ki-67 and CD31 staining by high-dose sulindac, with no significant changes in COX-1/COX-2 levels or activity in any of the treatment groups. NF- κ B DNA-binding activity was significantly decreased by high-dose LC-1. Cyclin D1 protein levels were reduced by the low-dose LC-1/sulindac combination or high-dose sulindac alone, correlating with BxPC-3 tumor suppression. These results suggest that LC-1 and sulindac may mediate their antitumor effects, in part, by altering cyclin D1 levels. Furthermore, this study provides preclinical evidence for the therapeutic efficacy of these agents. [Mol Cancer Ther 2007;6(6):1736–44]

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States (1). Due to its elusive and aggressive nature, pancreatic cancer is difficult to detect at an early stage, resulting in a 5-year survival rate of <5% and a median survival time of <6 months. At the time of diagnosis, surgical resection is unfortunately not an option for many patients. Moreover, current chemotherapeutic strategies have limited effectiveness due to the innate chemoresistance of pancreatic carcinomas. Novel therapies are needed to counteract the signaling pathways that promote growth and resistance in pancreatic cancer.

Proliferative and survival pathways that may play a key role during various stages of pancreatic tumorigenesis include the mitogen-activated protein kinase/extracellular signal-regulated kinase, Akt, and nuclear factor- κ B (NF- κ B) cascades (2–4). The epidermal growth factor receptor is also frequently overexpressed in pancreatic cancer, which may contribute to the aberrant activation of cellular signaling pathways (5–7). Targeted molecular therapies to suppress these pathways have been of recent interest (8, 9). In addition, combination therapies with agents that target multiple pathways or with conventional therapies, such as the chemotherapeutic drug gemcitabine (2',2'-difluorodeoxycytidine), are currently being pursued (10). Such combinations may improve efficacy by providing synergism as well as minimizing toxicity by the use of lower doses of the single agents.

Successful therapies must overcome both innate and acquired resistance induced by exposure to chemotherapeutic agents or radiation (11, 12). Activation of the NF- κ B pathway is involved in resistance as well as in cell growth and survival (13–15). We previously reported that parthenolide, a sesquiterpene lactone isolated from the medicinal herb feverfew (*Tanacetum parthenium*), effectively targeted the NF- κ B pathway and inhibited pancreatic cancer cell growth *in vitro* (16). In addition, the combination of parthenolide and the cyclooxygenase (COX) inhibitor

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Requests for reprints: C. Max Schmidt or Michele T. Yip-Schneider, Department of Surgery, Indiana University School of Medicine, 1044 West Walnut Street, Building R4, Room 041, Indianapolis, IN 46202. Phone: 317-278-4739; Fax: 317-274-0396. E-mail: maxschmi@iupui.edu or myipschn@iupui.edu

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sulindac synergistically inhibited the growth of two different human pancreatic cancer cell lines, BxPC-3 and MiaPaCa-2, and cooperatively targeted the NF- κ B pathway (16). The combination was effective in BxPC-3 cells, which express both COX-1 and COX-2, as well as in MiaPaCa-2 cells, which lack COX; thus, the inhibitory effects of the combination were determined to be at least in part COX independent. These results show the efficacy of combination therapy with parthenolide and sulindac *in vitro* and their potential for the treatment of pancreatic cancer *in vivo*.

Recently, a derivative of parthenolide with improved bioavailability, designated LC-1, was synthesized. In the present study, we compared the inhibitory activities of LC-1 and parthenolide *in vitro*. We also evaluated the efficacy of LC-1 and sulindac *in vivo* using a xenograft model of human pancreatic cancer. High-dose sulindac alone and low-dose combination treatment with LC-1/sulindac effectively suppressed pancreatic tumor growth. Ki-67 and CD31 staining were decreased by high-dose sulindac, reflecting inhibition of proliferation and angiogenesis, respectively. Furthermore, we showed that tumor suppression by high-dose sulindac alone and low-dose combination treatment with LC-1/sulindac was associated with reduced expression of the cell cycle regulatory protein cyclin D1.

Materials and Methods

Cell Culture and Treatments

BxPC-3 and MiaPaCa-2 human pancreatic cancer cells were obtained from the American Type Culture Collection and maintained as recommended. Parthenolide (Sigma-Aldrich) was dissolved in ethanol (40 mmol/L stock) and stored at -20°C . LC-1 (kindly provided by Dr. P.A. Crooks, University of Kentucky, Lexington, KY) was dissolved in water and stored at -20°C . Sulindac (Sigma-Aldrich) was dissolved in DMSO and stored at -20°C . Recombinant human tumor necrosis factor- α (R&D Systems) was dissolved in PBS containing 0.1% bovine serum albumin (10 $\mu\text{g}/\text{mL}$) and stored at -20°C .

Cell Growth

MiaPaCa-2 cells were plated in triplicate in 96-well plates. The next day, increasing concentrations of parthenolide or LC-1 were added (day 1). On day 3, cell growth was determined by the addition of CellTiter 96 Cell Proliferation reagent (Promega), and the absorbance was read at 450 nm. Cell growth was determined relative to the growth of control treated cells (100%). Alternatively, cells were plated in six-well plates and trypan blue-excluded cell counts were done in duplicate. Cell growth was expressed relative to control treated cells (100%).

Electrophoretic Mobility Shift Assay

Cells were plated in six-well plates and grown to 50% to 70% confluency. Cells were incubated with parthenolide or LC-1 at the indicated concentrations for 3 h. Cells were treated with tumor necrosis factor- α (5 ng/mL) for 10 min before harvesting. Whole-cell lysates were prepared and

incubated with radiolabeled probes specific for NF- κ B or Oct-1 (Promega) as the internal control as described previously (17). DNA-protein complexes were separated by electrophoresis and visualized by autoradiography.

NF- κ B (p65) ELISA

Whole-cell extracts were prepared from representative tumors according to the manufacturer's protocol (Active Motif). NF- κ B DNA-binding activity was measured using the TransAM p65 NF- κ B ELISA-based kit as recommended (Active Motif).

Ex vivo Prostaglandin E₂ Analysis

Athymic nude mice (Harlan) were injected s.c. with BxPC-3 cells (2×10^6). When tumors reached ~ 1 cm in diameter, a single dose of sulindac or placebo was given by oral gastric lavage ($n = 3$ per group). Tumors were harvested 9 or 20 h after treatment, frozen in liquid nitrogen, and stored at -80°C . At the time of analysis, tumors were minced and homogenized in lysis buffer [0.05 mol/L Tris (pH 7.4) containing 10 $\mu\text{g}/\text{mL}$ indomethacin]. Tumor homogenates (3,000 μg total protein) in 0.5 mL lysis buffer were mixed with equal volume water/ethanol (1:4) and 10 μL glacial acetic acid and spun (2,500 $\times g$, 2 min). Supernatants were loaded onto primed C18 Amprep columns (Amersham Biosciences). Samples were eluted with ethyl acetate and evaporated under nitrogen. Samples were reconstituted in assay buffer [supplied in prostaglandin E₂ (PGE₂) kit, Amersham Biosciences] for PGE₂ analysis. The PGE₂ ELISA is based on competition between unlabeled PGE₂ and a fixed quantity of peroxidase-labeled PGE₂ for binding to a PGE₂-specific antibody bound to a plate. The amount of the bound PGE₂ peroxidase is measured by the addition of the substrate. Results are expressed as pg PGE₂ per μg total protein.

Xenograft Model

The following animal study is in compliance with federal Institutional Animal Care and Use Committee guidelines. Athymic nude mice were injected s.c. with BxPC-3 or MiaPaCa-2 cells (2×10^6) and randomly assigned to treatment groups: placebo (10% Cremophor EL, 10% ethanol, and 80% water), low-dose/high-dose LC-1 (10 or 20 mg/kg and 40 mg/kg), low-dose/high-dose sulindac (20 or 60 mg/kg), and low-dose combination of LC-1/sulindac (10 or 20 mg/kg and 20 mg/kg, respectively). Treatment was initiated on the day of injection (day 1) and given once daily by oral gastric lavage. Tumors were measured twice weekly and mean tumor volume for each treatment group was calculated according to the formula $[(d1 + d2) / 4]^2 \pi h$. The mice were sacrificed on day 37 (BxPC-3) or day 43 (MiaPaCa-2), and tumors were excised. Tumor sections were either frozen directly in liquid nitrogen for storage at -80°C or fixed in 10% formalin (Sigma) for 48 h. Fixed tissues were then transferred to 70% ethanol and embedded in paraffin, and serial sections (5 μm) were cut for immunohistochemistry.

Immunohistochemistry

Slides were deparaffinized, hydrated, and placed in antigen retrieval citrate buffer (pH 6.0; Dako North America) in a pressure cooker for 15 min. For Ki-67

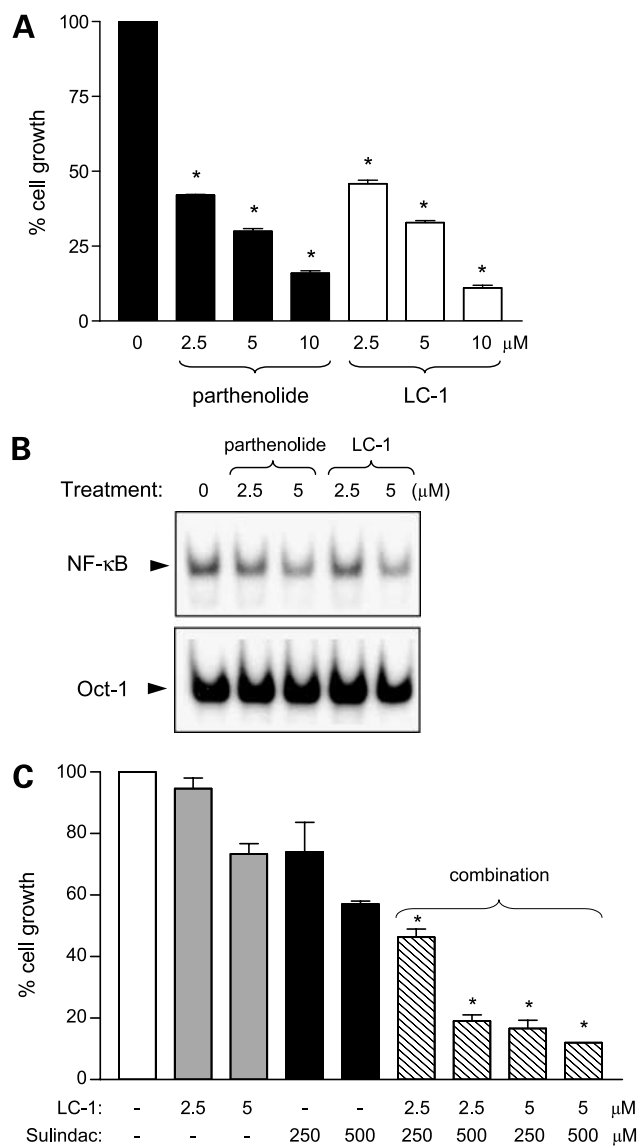


Figure 1. Inhibitory activity of parthenolide and LC-1 *in vitro*. **A**, cell growth. MiaPaCa-2 cells were treated with the indicated concentrations of parthenolide or LC-1 for 72 h. Percentage cell growth was determined by a colorimetric proliferation assay and expressed relative to control treated cells (100%). *Columns*, mean from a representative experiment set up in triplicate; *bars*, SE. *, $P < 0.05$ versus placebo. **B**, NF-κB DNA-binding activity. Extracts were prepared from MiaPaCa-2 cells treated with either parthenolide or LC-1 for analysis by electrophoretic mobility shift assay with NF-κB-specific or Oct-1-specific (internal control) probes. **C**, combination treatment with LC-1/sulindac. MiaPaCa-2 cells were treated with LC-1 and/or sulindac for 72 h. Cell growth was determined by cell counts and expressed relative to control treated cells (100%). *Columns*, mean from two independent experiments counted in duplicate; *bars*, SE. *, $P < 0.05$ versus single agents.

staining, slides were blocked with avidin/biotin (Dako) for 10 min. Slides were then placed in 3% H₂O₂ for 10 min followed by incubation in protein block (Dako) for 15 min. Slides were stained with Ki-67 (mouse anti-rat, 1:50; Dako) or cyclin D1 primary antibodies (SP4, 1:100 dilution;

NeoMarkers) for 1 h followed by incubation with the secondary antibody and counterstaining. Ki-67-positive cells were counted in four random fields (×400 magnification). Cyclin D1-positive cells were counted in two fields with the highest density of staining (×400 magnification). All slides were counted independently by two investigators blinded to the treatment groups.

For CD31 staining, sections were prepared from frozen tissues. After air drying, slides were fixed with acetone/ethanol (70%/30%) for 10 min at 4°C. Slides were rinsed and processed as above from the H₂O₂ step. Slides were stained overnight with CD31 antibody (rat anti-mouse, 1:500; BD Pharmingen). CD31-positive microvessels were counted in three fields (×200) with the highest density of microvessels. Individual endothelial cells or clusters of endothelial cells were considered individual vessels.

Western Blotting Analysis

Cells were lysed in radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1%

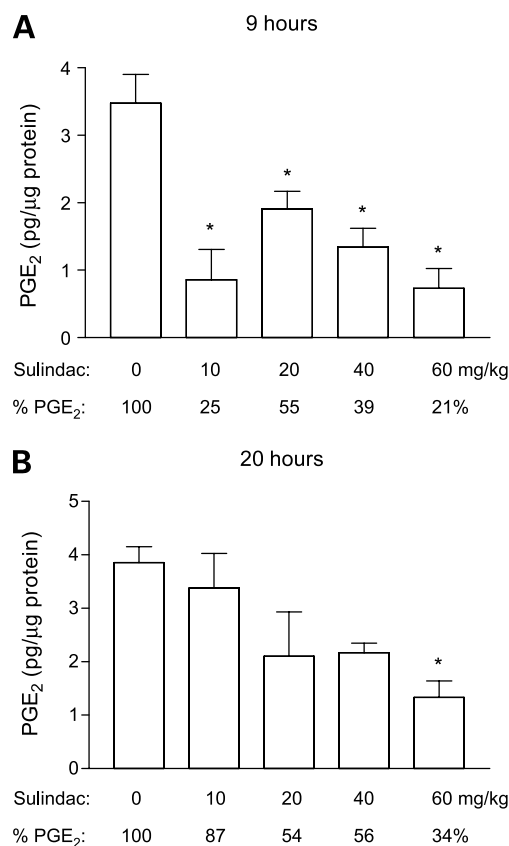


Figure 2. Effect of sulindac on PGE₂ levels *ex vivo*. PGE₂ levels were determined *ex vivo* using nude mice bearing BxPC-3 pancreatic flank tumors. Nine or 20 h after a single oral dose of sulindac, tumors were harvested for analysis. Intratumoral PGE₂ levels were determined by ELISA. Percentage PGE₂ following treatment with the indicated doses of sulindac relative to placebo (100%). *Columns*, mean ($n = 3$ /group); *bars*, SE. *, $P < 0.05$ versus control.

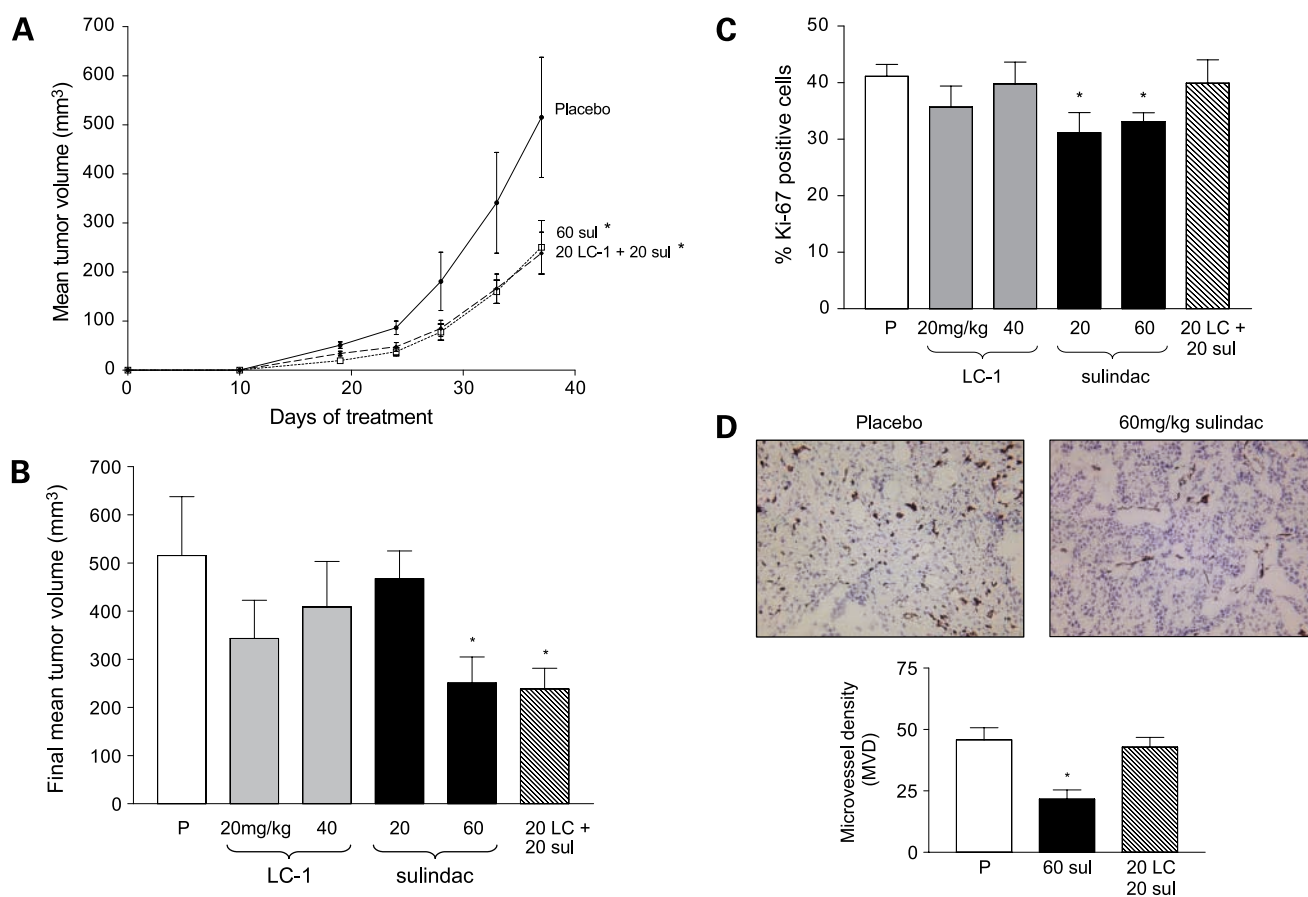


Figure 3. Effect of LC-1 and sulindac on BxPC-3 tumor growth *in vivo*. **A**, tumor growth of BxPC-3 xenografts. Nude mice were injected with BxPC-3 cells and randomized to treatment groups ($n = 6/\text{group}$). Effect of low-dose LC-1/sulindac (20 LC-1 + 20 sul, dashed line) and high-dose sulindac (60 sul, dotted line) versus placebo (solid line) on mean tumor volume over time. Points, mean; bars, SE. *, $P < 0.05$ versus placebo. **B**, final mean tumor volume of BxPC-3 xenografts. Final mean tumor volume of placebo (P) and each of the treatment groups. *, $P < 0.05$ versus placebo. **C**, Ki-67 staining. The percentage of Ki-67 positive cells was determined in four random fields (400 \times) for placebo and the treatment groups. Columns, mean ($n = 3/\text{group}$); bars, SE. *, $P < 0.05$ versus placebo. **D**, CD31 staining. CD31-positive microvessels were counted in three fields (200 \times) with the highest density of microvessels. Representative sections from the placebo and high-dose sulindac groups. Columns, mean microvessel density (MVD; $n = 3/\text{group}$); bars, SE. *, $P < 0.05$ versus placebo.

SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ aprotinin, 1 mmol/L Na_3VO_4 , and the supernatants were obtained. Cell lysates (10 μg total protein) were resolved by SDS-PAGE on 4% to 20% gradient gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore). The blots were probed with antibodies specific for COX-1 or actin (Santa Cruz Biotechnology), COX-2 (Cayman Chemical), and cyclin D1 (NeoMarkers) according to the manufacturer's protocol followed by enhanced chemiluminescence detection (Perkin-Elmer Life Sciences). Relative expression was determined by densitometry and expressed relative to the average band intensity of the placebo group (Scion Image, NIH).

Statistics

Xenograft tumor growth was analyzed by a random effects exponential regression. Statistical analysis of the treatment effect was done with $P < 0.05$ considered significant. Other comparisons between treatment and

placebo groups were analyzed by ANOVA with Dunnett's post test except when the Student's t test was done as indicated (Prism 3.02 software, GraphPad). $P < 0.05$ was considered significant.

Results

Inhibitory Activity of Parthenolide and LC-1 *In vitro*

To determine whether the derivative LC-1 and parthenolide possess similar *in vitro* activities, effects on cell growth and the NF- κ B pathway were compared in the human pancreatic cancer cell line MiaPaCa-2 (Fig. 1). As shown by proliferation and electrophoretic mobility shift assays, LC-1 and parthenolide showed very similar abilities to inhibit cell growth and NF- κ B DNA-binding activity, respectively (Fig. 1A and B). Furthermore, LC-1 in combination with sulindac cooperatively inhibited MiaPaCa-2 cell growth as shown previously with parthenolide

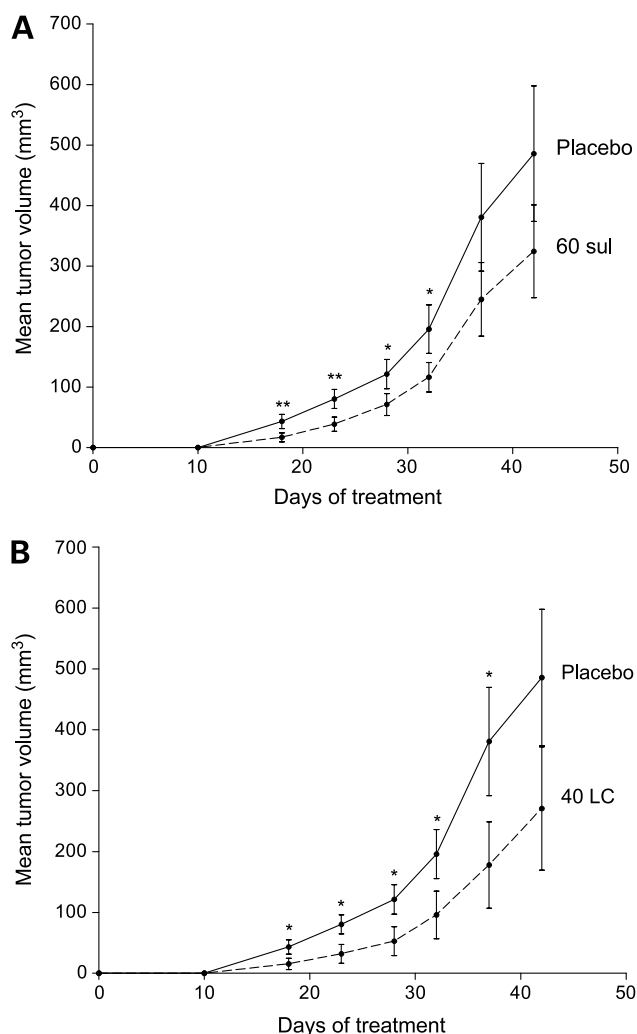


Figure 4. Effect of LC-1 and sulindac on MiaPaCa-2 tumor growth *in vivo*. **A**, MiaPaCa-2 xenografts treated with sulindac. Effect of high-dose sulindac (60 sul) on mean tumor volume over time. Points, mean ($n = 9$ /group); bars, SE. **, $P < 0.05$ versus placebo; *, $0.05 < P < 0.06$ versus placebo (t test). **B**, MiaPaCa-2 xenografts treated with LC-1. Effect of high-dose LC-1 (40 LC) on mean tumor volume over time. Points, mean ($n = 9$ /group); bars, SE. *, $P < 0.05$ versus placebo (t test).

(Fig. 1C; ref. 16). For example, 2.5 $\mu\text{mol/L}$ LC-1 resulted in $\sim 5\%$ growth inhibition, 500 $\mu\text{mol/L}$ sulindac resulted in $\sim 45\%$ inhibition, and the combination of these two doses of drugs resulted in $>80\%$ inhibition of cell growth. Thus, the derivative LC-1 and parthenolide exhibit equivalent *in vitro* activities associated with inhibition of the NF- κ B pathway in pancreatic cancer cells. With its improved bioavailability, LC-1 was chosen for evaluation *in vivo* either alone or in combination with sulindac.

Ex vivo Analysis of PGE₂ Levels following Exposure to Sulindac

The nonsteroidal anti-inflammatory drug sulindac inhibits the enzyme COX, which catalyzes the reaction leading to the production of PGE₂. To determine the effective dose of

sulindac for *in vivo* studies, PGE₂ levels in xenografted BxPC-3 pancreatic flank tumors were determined following a single oral dose of sulindac. Nine or 20 h after sulindac administration, tumors were harvested for analysis. The effect of various doses of sulindac on intratumoral PGE₂ levels *ex vivo* was measured by ELISA. Sulindac doses of 20 and 60 mg/kg reduced PGE₂ levels to 54% and 34% of placebo levels, respectively, for up to 20 h, showing that these doses of sulindac are capable of suppressing PGE₂ levels *in vivo* (Fig. 2A and B).

Effect of LC-1 and Sulindac on Pancreatic Tumor Growth *In vivo*

The efficacy of LC-1 and sulindac was evaluated in xenografts generated from either the COX-positive human pancreatic cancer cell line BxPC-3 or COX-negative MiaPaCa-2 cells. Nude mice were injected s.c. with BxPC-3 ($n = 6$ per group) or MiaPaCa-2 ($n = 9$ per group) cells and randomized to the following treatment groups: placebo, low-dose/high-dose LC-1 (low dose of 10 mg/kg for MiaPaCa-2 or 20 mg/kg for BxPC-3, high dose of 40 mg/kg; doses previously determined by C.J. Sweeney),⁹ low-dose/high-dose sulindac (20 or 60 mg/kg), or low-dose LC-1/sulindac combination (10 or 20 mg/kg and 20 mg/kg, respectively). Because MiaPaCa-2 cells were previously shown to be more sensitive to parthenolide *in vitro*, a lower dose of 10 mg/kg was given for the low-dose LC-1 treatment (16). Treatment was initiated on the day of injection and given once daily by oral gastric lavage for the length of the study.

BxPC-3 tumors were detectable within 2 weeks of injection in 100% of the mice. Over the course of the study, the low-dose LC-1/sulindac combination or high-dose 60 mg/kg sulindac groups had significantly smaller mean tumor volumes compared with placebo (Fig. 3A). The final mean tumor volumes were reduced $\sim 50\%$ in these two treatment groups relative to placebo (Fig. 3B). Treatment with LC-1 alone or low-dose sulindac alone did not significantly affect tumor size. These results show that treatment with high-dose 60 mg/kg sulindac alone or with the low-dose combination of LC-1/sulindac (20 mg/kg each) effectively suppressed pancreatic tumor growth relative to placebo. Immunohistochemical analysis of the BxPC-3 tumor sections stained with Ki-67 to measure proliferative index showed a significant decrease in Ki-67 staining in the sulindac groups compared with placebo (Fig. 3C). Similarly, CD31 staining to quantitate microvessel density was significantly decreased by high-dose sulindac (Fig. 3D). These results suggest that tumor growth suppression by sulindac may be mediated, in part, by decreasing proliferation and angiogenesis; however, similar effects were not observed in the combination treatment group. No significant difference in apoptosis was observed between treatment groups (data not shown).

In the COX-negative MiaPaCa-2 xenografts, tumor formation was delayed in the 40 mg/kg LC-1 group

⁹ Personal communication.

relative to placebo. Three weeks after injection, only 44% (four of nine total) of the LC-1–treated mice had formed tumors versus 89% (eight of nine total) in the placebo group; by the end of the study, tumor formation efficiency was 78% (seven of nine total) in the LC-1 group and 89% (eight of nine total) in the placebo group. Treatment of the MiaPaCa-2 xenografts with high-dose 60 mg/kg sulindac or 40 mg/kg LC-1 alone decreased tumor volumes at multiple time points to a significant degree (Fig. 4A and B). The LC-1/sulindac combination had no effect on tumor growth (data not shown).

Effect of Sulindac and LC-1 on Target Pathways COX and NF- κ B in BxPC-3 Xenografts

Representative BxPC-3 tumor lysates were prepared from the low-dose single agents, combination, and high-dose sulindac groups for analysis. No difference in either COX-1 or COX-2 protein levels was observed between treatment groups (Fig. 5A). Similarly, COX activity as measured by intratumoral PGE₂ levels was not significantly affected by any of the drug treatments (Fig. 5B). The lack of effect on either COX expression or activity suggests that the agents are mediating their effects in a COX-independent manner.

To evaluate the NF- κ B pathway, whole-cell extracts were prepared from representative BxPC-3 tumors and NF- κ B-binding activity was measured by a p65 NF- κ B ELISA. NF- κ B-binding activity was significantly decreased in the high-dose 40 mg/kg LC-1 group relative to placebo, confirming that LC-1 is targeting the NF- κ B pathway (Fig. 5C). No effect on binding was apparent in any of the other treatment groups.

Tumor Suppression Correlates with Inhibition of Cyclin D1 Expression

Overexpression of cyclin D1, a regulatory protein required for cell cycle progression, in pancreatic tumors has been associated with poor prognosis (18). To determine whether the level of cyclin D1 expression correlated with the inhibitory effect of the low-dose LC-1/sulindac combination or high-dose sulindac, BxPC-3 tumor lysates were prepared from these treatment groups. Cyclin D1 protein levels were significantly decreased in both the low-dose LC-1/sulindac and high-dose sulindac groups, thus corresponding with tumor suppression (Fig. 6A). In the case of sulindac, decreased proliferation in the BxPC-3 xenografts correlates with the observed decrease in cyclin D1 levels. These results were confirmed by immunohistochemistry showing fewer cyclin D1–positive cells in the low-dose LC-1/sulindac and high-dose sulindac groups relative to placebo (Fig. 6B).

Discussion

The rates of incidence and mortality are nearly identical for pancreatic cancer. Despite advances in surgery, radiotherapy, and chemotherapy, >90% of patients with pancreatic cancer die of chemoresistant disease. Targeted or novel combination treatment strategies may offer improved responses to conventional therapies by increasing specific-

ity of inhibition toward cancer cells and reducing toxicity toward normal cells. Potential targets of interest include proteins or cellular pathways that are aberrantly expressed or activated in pancreatic cancer.

The activity of the transcription factor NF- κ B and the expression of the arachidonic acid pathway enzyme COX-2 are up-regulated in pancreatic cancer (19, 20). Activation of these pathways may promote cell growth, invasion, and chemoresistance as well as suppress apoptosis during various stages of pancreatic tumorigenesis (13, 15, 21–23).

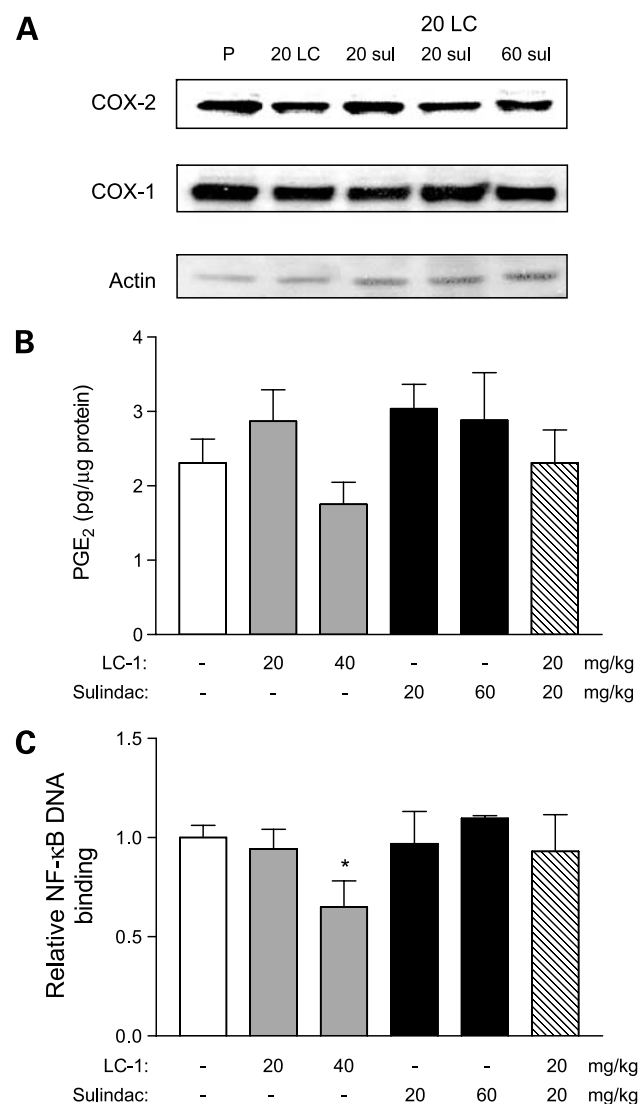


Figure 5. Target pathway analysis of BxPC-3 xenograft tumors. **A**, COX expression. Representative lysates were prepared from placebo and the indicated treatment groups for analysis by Western blot to detect COX-2, COX-1, and actin protein levels. **B**, PGE₂ levels. PGE₂ levels were measured by ELISA in representative tumors from the treatment groups. **C**, NF- κ B activity. Extracts were prepared from representative tumors, and NF- κ B DNA-binding activity was determined by ELISA. Binding was expressed relative to control treated cells. **Columns**, mean ($n = 6$ /group); **bars**, SE. *, $P < 0.05$ versus placebo.

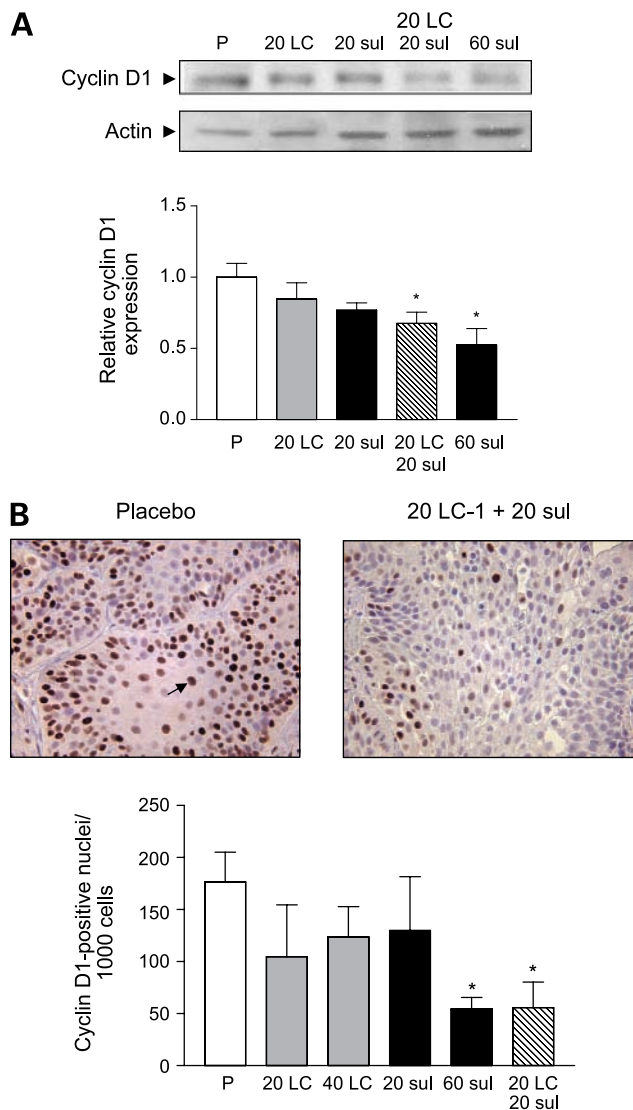


Figure 6. Effect on cyclin D1 expression. **A**, Western blot analysis. Lysates were prepared from representative BxPC-3 tumors and analyzed by Western blot to detect cyclin D1 expression. *Bottom*, relative cyclin D1 expression is expressed graphically. *Columns*, mean ($n = 4-6$ /group); *bars*, SE. *, $P < 0.05$ versus placebo. **B**, cyclin D1 immunohistochemistry. Tumor sections were stained for cyclin D1, and representative sections from the placebo and low-dose LC-1/sulindac combination groups are shown. Original magnification, $\times 400$. Cyclin D1-positive cells in two of the most densely stained $400\times$ fields ($\sim 1,000$ cells total) were counted on each slide. *Columns*, mean ($n = 5$ /group); *bars*, SE. *, $P < 0.05$ versus placebo.

We have targeted these two pathways *in vitro* with parthenolide, a proapoptotic drug associated with NF- κ B inhibition, and nonsteroidal anti-inflammatory drugs known to inhibit COX. In human pancreatic cancer cell lines, we previously showed complementary growth inhibition with the combination of parthenolide and the COX inhibitor sulindac (16). The effects were mediated in a COX-independent manner, correlating with suppression of the NF- κ B pathway in both COX-positive (BxPC-3) and

COX-negative (MiaPaCa-2 and PANC-1) pancreatic cancer cell lines. Based on these promising *in vitro* results, a recently synthesized water-soluble derivative of parthenolide, LC-1, was evaluated with sulindac *in vivo* in the present study.

Xenografts were generated from the COX-positive pancreatic cancer cell line BxPC-3 or the COX-negative MiaPaCa-2 cells to evaluate the antitumor effects of LC-1 and/or sulindac. Treatment of the MiaPaCa-2 xenografts with high-dose LC-1 alone resulted in delayed tumor formation. In addition, the high doses of LC-1 or sulindac alone showed a decrease in weekly tumor volumes. The LC-1/sulindac combination had no effect, possibly due to the lower LC-1 dose (10 mg/kg) used. These results suggest that, although these agents may have some efficacy in the treatment of COX-negative pancreatic cancers, the tumors may eventually be able to resist and overcome the inhibitory effects. Interestingly, the COX-2 inhibitor nimesulide was previously shown to reduce tumor angiogenesis and growth in a COX-2-positive pancreatic cancer; in contrast, the opposite effect was observed in a COX-2-negative pancreatic cancer (24).

Treatment of the COX-positive BxPC-3 xenografts with either the low-dose combination LC-1/sulindac or high-dose sulindac alone significantly suppressed tumor growth relative to placebo. Ki-67 and CD31 staining were significantly decreased in the high-dose sulindac group, suggesting that tumor growth suppression by sulindac may be mediated by inhibiting proliferation and angiogenesis. Only the high-dose LC-1 group showed a significant decrease in NF- κ B DNA-binding activity. However, this partial NF- κ B inhibition did not translate into significant growth inhibition of BxPC-3 tumors. This contrasts with our previous *in vitro* findings that showed an association between NF- κ B pathway inhibition and growth suppression by parthenolide and/or sulindac (16). For the low-dose combination treatment group, other targets may be involved in mediating the inhibitory effects of LC-1. Possibilities include the c-Jun NH₂-terminal kinase pathway because the parent compound parthenolide has recently been shown to reverse the resistance of breast cancer cells by activating c-Jun NH₂-terminal kinase (25).

No significant change in COX-1/COX-2 protein expression or COX activity as measured by PGE₂ production was observed in any of the treatment groups, suggesting a COX-independent mechanism of action. The apparent lack of long-term inhibition of COX activity by sulindac, despite observed suppression of this pathway both *ex vivo* and *in vitro*, raises the possibility of acquired resistance (i.e., efflux pumps preventing drug from getting to target). Although initially responsive to the agents, following repeated exposure, the known targets may lose sensitivity to inhibition. Nevertheless, decreased expression of cyclin D1 did correlate with tumor suppression by the low-dose combination LC-1/sulindac or high-dose sulindac alone, suggesting that the inhibitory effects of these agents may be mediated by altering cyclin D1 levels, thus identifying cyclin D1 as a common target.

The importance of the down-regulation of cyclin D1 is underscored by the fact that it is a critical cell cycle regulatory protein, required for progression through G₁ to S phase (26, 27). In pancreatic cancer, cyclin D1 overexpression is associated with poor prognosis and decreased postoperative survival (18, 28). Inhibition of cyclin D1 expression by cyclin D1 antisense suppressed pancreatic cell growth and tumorigenicity, identifying cyclin D1 as an important regulator in pancreatic cells (29). In addition, we have previously reported decreased cyclin D1 expression following treatment of COX-positive and COX-negative pancreatic cancer cells with a panel of nonsteroidal anti-inflammatory drugs, including sulindac, which inhibit growth and cell cycle progression (30). More recently, the role of cyclin D1 has been investigated in an elastase-myc pancreatic cell line (31). Cyclin D1 overexpression induced cell proliferation and anchorage-independent cell growth and exhibited resistance to treatment with cisplatin. In contrast, inhibition of cyclin D1 expression by small interfering RNA resulted in increased sensitivity to cisplatin-induced apoptosis. These results suggest that cyclin D1 may contribute to the chemoresistance of pancreatic cancer cells by increasing cell proliferation and decreasing apoptosis. Thus, our observation of reduced tumor size following LC-1/sulindac combination or high-dose sulindac treatments may be mediated by decreased production of cyclin D1, resulting in greater chemosensitivity.

The promoter region of cyclin D1 contains binding sites for several transcription factors, including response elements for NF- κ B, SP1, Oct-1, cyclic AMP-responsive element, and E2F (32). Although high-dose LC-1 decreased NF- κ B DNA-binding activity, the other treatments did not, suggesting that the ability of LC-1/sulindac and sulindac alone to decrease cyclin D1 levels is not mediated by NF- κ B. Furthermore, cyclin D1 inhibition by sulindac seems to be COX independent because no decrease in the level of PGE₂ was detected. Further work is required to determine the mechanism of cyclin D1 down-regulation.

Based on the results from this study, it seems that the use of a low-dose combination of the bioavailable inhibitor LC-1 and sulindac or high-dose sulindac alone may represent a promising strategy for the development of more effective therapies for the treatment of pancreatic cancer. Suppression of pancreatic tumor growth by these agents was shown to be independent of COX and NF- κ B but was associated with inhibition of cyclin D1 expression. Taken together, these results identify cyclin D1 as a central growth regulator in pancreatic cancer cells and an important determinant of response to therapy. Further studies to define the mechanism of action of these agents will facilitate optimization of therapy as well as provide insight into pathways of resistance.

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