Ormeloxifene Suppresses Desmoplasia and Enhances Sensitivity of Gemcitabine in Pancreatic Cancer

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Abstract

The management of pancreatic ductal adenocarcinoma (PDAC) is extremely poor due to lack of an efficient therapy and development of chemoresistance to the current standard therapy, gemcitabine. Recent studies implicate the intimate reciprocal interactions between epithelia and underlying stroma due to paracrine Sonic hedgehog (SHH) signaling in producing desmoplasia and chemoresistance in PDAC. Herein, we report for the first time that a nonsteroidal drug, ormeloxifene, has potent anticancer properties and depletes tumor-associated stromal tissue by inhibiting the SHH signaling pathway in PDAC. We found that ormeloxifene inhibited cell proliferation and induced death in PDAC cells, which provoked us to investigate the combinatorial effects of ormeloxifene with gemcitabine. Ormeloxifene potentiated the antitumorigenic effect of gemcitabine by 75% in PDAC xenograft mice. Furthermore, ormeloxifene depleted tumor-associated stroma in xenograft tumor tissues by inhibiting the SHH cellular signaling pathway and mouse/human collagen I expression. Xenograft tumors treated with ormeloxifene in combination with gemcitabine restored the tumor-suppressor miR-132 and inhibited stromal cell infiltration into the tumor tissues. In addition, invasiveness of tumor cells cocultivated with TGFβ-stimulated human pancreatic stromal cells was effectively inhibited by ormeloxifene treatment alone or in combination with gemcitabine. We propose that ormeloxifene has high therapeutic index and in a combination therapy with gemcitabine, it possesses great promise as a treatment of choice for PDAC/pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis largely due to its propensity for early local invasion, distant metastasis, and lack of effective therapies. Many chemotherapeutic regimens have failed and the current standard-of-care therapy, gemcitabine, extends patient survival by only a few months (1). Newer treatment options for PDAC patients are FOLFIRINOX and nab-paclitaxel/gemcitabine, which improved overall survival by 4.3 and 1.8 months over gemcitabine therapy, respectively (2,3).

In addition, safety profile of these drugs is less favorable than gemcitabine therapy, accounting for myelosuppression and peripheral neuropathy (2–4). Despite these advances, the overall outcome remains miserable for this patient population. Thus, investigations on alternative approaches for PDAC therapy are a high research priority.

Activation of oncogenes, such as Kras, and/or inactivating mutations or loss of expression of tumor-suppressor genes (including DPC4, p16, p53, and SMAD4) is known in PDAC (5). It has been shown that extensive desmoplasia is one of the underlying causes of pancreatic cancer’s poor prognosis and chemoresistance (6). Desmoplasia is typically characterized by excessive production of extracellular matrix (ECM) and collagen I and associated with proliferation of stromal cells, myofibroblasts, and pancreatic stellate cells. A profound role of Sonic hedgehog (SHH) pathway is implicated in desmoplasia (7) and cancer progression (8), including PDAC (9). This developmental pathway, dormant in the adult pancreas, becomes reactivated early in PDAC development (10). SHH is a member of the Hedgehog (Hh) family of secreted signaling proteins, having diverse functions during vertebrate development (11). In pancreatic tumors, intimate reciprocal interactions occur between epithelia and underlying stroma due to paracrine Hh signaling that lead to desmoplasia and form a barrier to chemotherapy drug(s) penetration (12). Depletion of tumor stroma leads to the increasing functional vasculature that provides a feasible avenue for efficient therapeutic drug delivery (13). In addition, Hh signaling...
plays a key role in the maintenance of pancreatic cancer stem cells (CSC) that are involved in drug resistance, cancer recurrence, and poor clinical outcome (10). Therefore, molecular and/or chemical intervention to target Hh signaling and disruption in the microenvironment in tumors could be an interesting therapeutic approach for pancreatic cancer (13). Some of the well-known Hh signaling antagonists, such as vismodegib (GDC-0449), have been investigated alone or as an adjuvant to the traditional anticancer drugs but have not yielded clinically meaningful results (14, 15) and have shown notable adverse effects including teratogenic properties (16, 17). Thus, identification of novel therapies with high therapeutic index that can target Hh and tumor progression signaling pathways with no or minimal adverse effects is required.

Repurposing of established drugs as anticancer agents is a current active investigative approach. Ormeloxifene is a non-hormonal, non-steroidal (oral) contraceptives molecule. Recent studies suggested that ormeloxifene may be effective in inhibiting breast cancer, head and neck cancer, and chronic myeloid leukemia cells (18). Moreover, ormeloxifene is reported to have an excellent therapeutic index and is safe for chronic administration (19). This study demonstrates the inhibitory role of ormeloxifene on the SHH signaling pathway, and describes inhibitory patterns of this drug on pancreatic tumor progression using bidirectional tumor stromal interactions. This inhibitory effect was either more pronounced or comparable with a known smoothened (SMO) inhibitor, GDC-0449, in PDAC cells. Ormeloxifene disrupts the stroma of fibrotic pancreatic tumors and inhibits the proliferating stellate and myeloid cells involved in the development of pancreatic fibrosis. Furthermore, the combinatorial effects of ormeloxifene with gemcitabine induce increased gemcitabine sensitivity. In addition, these studies also suggest wide use of ormeloxifene in PDAC patients due to its intended safe use in fertile women, considering the teratogenic potential of other Hh pathway inhibitors such as cyclopamine and GDC-0449 (20, 21).

Materials and Methods

Cell culture, growth conditions, and treatments

Cell lines were purchased from the ATCC and were maintained at 37°C/5% CO2 in recommended growth medium with 10% FBS (RPMI, DMEM and DMEM/Ham’s F12; Hyclone Laboratories). Human CSCs (CD133+/CD44+/CD24-/ESA+) were obtained from Celprogen Inc. They were isolated from primary tumors and have been described previously (22). All cell lines were maintained in continuous exponential growth by twice a week passaging in cell type-specific media. Ormeloxifene was generously synthesized and provided by Fathi Halaweish (South Dakota State University, Brookings, SD) as described earlier (23). Gemcitabine was purchased from Sigma-Aldrich (cat. no. G6423) and GDC-0449 from Selleckchem (cat. no. S1082). Cells were treated with indicated doses of ormeloxifene, gemcitabine, and GDC-0449 after completely solubilized in ethanol, PBS, and DMSO, respectively.

Cell proliferation by MTS assay

The antiproliferative effect of ormeloxifene was determined after 48 hours using the CellTiter 96 AQueous One solution assay (cat. no. G5421; Promega) using a microplate reader (BioMate 3 UV-Vis spectrophotometer; Thermo Electron Corporation). Ethanol- or PBS-containing medium served as the vehicle control. In addition, the antiproliferative effect of ormeloxifene was determined at 24 and 48 hours using Cell Counting Kit-8 (Mayflower Bioscience) and the percentage viability of Panc-1 and BxPC-3 cells was determined after treatment with GDC-0449 and ormeloxifene. The antiproliferative effect of each treatment was calculated as a percentage of cell growth with respect to the vehicle control.

Cell proliferation by xCELLigence assay

PDAC cells (10,000 cells/well) were seeded in E-plate (Roche) following the xCELLigence Real Time Cell Analyzer (RTCA) DP instrument manual as provided by the manufacturer (Roche; ref. 24). After 24 hours, ormeloxifene or the vehicle control was added and the experiment was allowed to run for 100 hours. Average baseline cell index for ormeloxifene-treated cells compared with control cells was calculated for at least two measurements from three replicated experiments.

Flow cytometric analysis of apoptosis and necrosis

BxPC-3 and Panc-1 cells (1 × 10⁶) were treated for 24 hours with ormeloxifene (15 μmol/L) and gemcitabine (100 nmol/L) alone and in combination. Cells were stained with Annexin V-FITC and propidium iodide (PI). The apoptotic and necrotic populations were detected as described earlier (25). Cells were scanned in FL-1 (FITC) versus FL-2 (PI) channels and analyzed using an Accuri C6 flow cytometer (Accuri Cytometers, Inc.).

Cell-cycle analysis

Cells were exposed to ormeloxifene (15 μmol/L) and gemcitabine (100 nmol/L) alone or in combination for 24 hours and stained with Tefold Reagent containing PI (cat. no. P-4170; Sigma-Aldrich). Cells were analyzed with an Accuri C6 flow cytometer. Cells with hypodiploid DNA (content less than G0-G1) were deemed apoptotic (sub-G0-G1).

Dual-Luciferase reporter assay

Dual-Luciferase reporter assay was carried out to investigate the effect of treatments on Gli-1 and NF-kB transcriptional activity using a luciferase assay kit (cat. no. E2940; Promega) according to the manufacturer’s protocol. BxPC-3 and Panc-1 cells were transfected with luciferase reporter constructs [NF-kB, gift from Dr. Ajay Singh, Mitchell Cancer Institute, Fairhope, AL, Signal CGL Reporter (luc) Kit, cat. no. CCS-6030L; Qiagen] and treated with ormeloxifene and gemcitabine alone or in combination for 24 hours. The normalized luciferase activity was expressed as a ratio of firefly luciferase to Renilla luciferase units.

Indirect coculture of PDAC cells and pancreatic stromal cells

Human pancreatic small cell (PSC) fibroblasts and stellate cells were attained from an islet transplant program and maintained in CMRL-1066 medium (cat. no. 15110; Corning) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate at 37°C in humidified atmosphere containing 5% CO₂. Human PSCs (3 × 10⁵ cells/culture insert) were seeded into the culture inserts of 1.0-μm pore size (BD Biosciences) in CMRL-1066 media. On day 2, the culture inserts were placed into 6-well plates containing Panc-1 cells (0.8 × 10⁶ cells/well), followed by treatment with ormeloxifene (10 μmol/L) and gemcitabine (100 nmol/L) and incubated up to 2 days in DMEM medium. As previous studies have shown TGFβ to be a potent inducer of epithelial–mesenchymal transition (EMT) in several cancer cells including pancreatic cancer cells (26, 27), we used recombinant...
TGβ (2 ng/mL) to stimulate the stromal cells as a mediator of PSC-induced EMT in cells.

Clonogenic assay
For the clonogenic assay, 500 cells were treated with indicated concentrations of ormeloxifene for 12 days. The visible colonies (≥50 cells) were counted following hematoxylin staining (Fisher Scientific) and the percentage of colonies was calculated as compared with control, as described earlier (28).

Cell motility, migration, and invasion assays
Cell motility was analyzed with a Boyden’s chamber assay (28). For cell invasion assays, BD Biocoat Matrigel Invasion Chambers (BD Biosciences) were used as per the manufacturer’s instructions. After 48 hours of incubation, the invading cells were stained and counted in 10 fields of view. In addition, a wound-healing migration assay was also used to evaluate the effect of ormeloxifene on the migratory ability of cancer cells. The cell monolayer was scraped using a micropipette tip and 48 to 72 hours after treatment, the residual gap length was calculated from photomicrographs. To further confirm these findings, real-time migration and proliferation were performed by the xCELLigence system, which is an electrical impedance-based method that allows for the measurement of cell migration and proliferation in real-time (24). Briefly, 4 x 10^4 cells were seeded per chamber of cell invasion and migration (CIM) plate and the cells were analyzed in xCELLigence instrument at 37°C, 5% CO2 for migration and invasion assays.

Immunoblot analysis
Human PDAC cells (1 x 10^6) were treated with ormeloxifene (15 μmol/L) and gemcitabine (100 nmol/L) alone and in combination for 24 hours. Total cell lysates were prepared followed by immunoblotting for various indicated proteins as described earlier (25).

Reverse transcription–quantitative real-time PCR
Total RNA was extracted using TRIzol reagent (cat. no. AM 9738; Invitrogen) and integrity was checked with an RNA 6000 Nano Assay kit and 2100 Bioanalyzer (Agilent Technologies). The mRNA expression levels were determined by quantitative real-time PCR (qRT-PCR) using TaqMan PCR master mixture and TaqMan-specific probes (Applied Biosystems). The expression of genes was normalized to the 18S rRNA gene.

Tumorsphere assay
Pancreatic CSCs were plated on ultra-low attachment plates (Corning) at a density of 1 x 10^3/100 μL well/96-well plate and treated with ormeloxifene or GDC-0449 (2.5–10 μmol/L). The plates were allowed to grow for 7 days in 0.5% serum-free medium (Cellprogen) to form primary spheres. Following the incubation, the primary spheres were dissociated into single-cell suspension and plated at a density of 1 x 10^4/2 mL/6-well ultra-low attachment plate. Secondary spheres were counted after 7 to 10 days in culture.

In vivo tumor xenograft model
Six-week-old female athymic nude (nu/nu) mice (22–25 mg) were purchased from Charles River Laboratories International, Inc., and maintained in a pathogen-free environment. The mice were injected with BxPC-3 cells intraperitoneally (i.p.; 3 x 10^6) and (5 x 10^6) cells in 200 μL PBS/Matrigel subcutaneously. On day 15, the mice were treated with vehicle (ethanol), ormeloxifene (200 μg), gemcitabine (500 μg), or their combination via i.p. injections, three times a week, for 6 subsequent weeks. Mice were weighed twice a week to monitor their health and tumor growth. Tumor volume (V) was estimated from the length (l), width (w), and height (h) of the tumor using the formula V = \( \frac{1}{2} \times l \times w \times h \), as described previously (Supplementary Fig. S4; ref. 28). Forty-five days after the first drug injection, mice were euthanized and tumor burden (wt) and metastases were noted. The organs, including pancreas, were harvested and checked for metastases. The data were modeled with time (discrete), group (control, ormeloxifene, gemcitabine, and ORM+GEM), and the interaction between them. Primary analyses involved planned comparisons (separately for each time point) between control and ormeloxifene/gemcitabine versus ORM+GEM. Animal care was performed in accordance with institutional guidelines and all animal experiments were carried out using protocols approved by the Sanford Research Institutional Animal Care and Use Committee.

In situ hybridization for microRNA detection and expression
We detected the expression of miR-132 in formalin-fixed paraffin-embedded (FFPE) tissues of control and treated xenograft mice. We used an in situ hybridization technique and used a Biochain kit (cat. no. K2191050; Biochain IsHyb In Situ hybridization kit) as previously described (29). Briefly, tissues were hybridized with hybridization buffer and digoxigenin-labeled probe (EXIQON) at 45°C overnight followed by incubation with the AP-conjugated anti-digoxigenin antibody and NBT/BCIP (Pierce) and nuclear fast red counterstaining.

Immunofluorescence and immunohistochemical analyses
Immunofluorescence and IHC analyses were used to analyze the untreated and treated xenograft tumor tissues to detect changes associated with the expression of important proteins involved in tumor–stromal interactions as described previously (30). The slides were stained with specific antibodies following heat-induced antigen retrieval techniques and imaged using a laser scanning confocal microscope (Nikon TIRF) with a x200 Apochromat objective for immunofluorescence. For immunohistochemistry, the slides were stained using Biocare’s MACH4 Universal HRP-Polymer kit (Biocare Medical) and analyzed as previously described (29, 30).

Statistical analyses
Statistical significance of the studies was analyzed by the Student t test. Differences with P values of < 0.05 are considered significant. Tumor size values were examined at the day 50 point, using an ANOVA approach. Tests of main effects (differences between treatments) and contrasts were performed.

Results
Ormeloxifene treatment suppresses tumorigenic features of PDAC cells
Ormeloxifene was found to have an anticancer effect on all tested PDAC cells (Fig. 1A and Supplementary Fig. S1A). To confirm these results, we measured the growth in real time for
Figure 1. Determination of proliferation, clonogenicity, and cytotoxicity profiles of ormeloxifene in PDAC cells. A, structure of ormeloxifene (IUPAC name: 1-[2-[4-[(3S,4R)-7-methoxy-2,2-dimethyl-3-phenyl-chroman-4-yl]phenoxy]ethyl]pyrrolidine) and its effect on cell growth was monitored by the MTS assay for 48 hours and is shown as percentage (Supplementary Fig. S2A). B, effect of ormeloxifene on cell proliferation with respect to time was also confirmed by xCELLigence RTCA. C, clonogenic assay was performed to determine the ability of cells to form colonies (percentage inhibition) following treatment. Cells were photographed and counted using AlphaEaseFC (Alpha Imager HP AIC) software analysis tool. Bars represent mean ± SD; n = 3; *, P < 0.05; ***, P < 0.001.

duration of 100 hours using the xCELLigence system (Fig. 1B). This assay monitors cell growth in real time by measuring changes in electric impedance between two golden electrodes embedded in the bottom of the cell culture wells. The impedance, which is converted to a cell index value, is directly proportional to the number of cells and also reflects the cells’ viability, morphology, and adhesion strength (31). The growth curve, which is presented as a baseline cell index, showed that ormeloxifene significantly reduced the baseline cell index compared with the control cells (Fig. 1B). Furthermore, ormeloxifene treatment inhibited the clonogenic potential of PDAC cells (BxPC-3, Panc-1, AsPC-1, MiaPaca, and HPAF-II) as evident by the decreased number of colonies after ormeloxifene treatment (Fig. 1C). Moreover, ormeloxifene was also found to inhibit cellular motility (Fig. 2A) and invasion (Fig. 2B) of PDAC cells. The inhibition of the migratory ability of cells is demonstrated by wound-healing assay (Supplementary Fig. S1B) and cellular invasion by Matrigel invasion assay (Supplementary Fig. S1C), which was further confirmed using the xCELLigence method (Fig. 2C).

In addition, we sought to compare the anticancer potential of ormeloxifene with a known SMO inhibitor (GDC-0449) in human PDAC cells. Ormeloxifene showed more pronounced or comparable inhibitory effect on cell proliferation, clonogenicity, and invasion than GDC-0449 at equal indicated concentrations (Supplementary Fig. S2A–S2C). Inhibition of cell viability and invasion was observed within 48 hours following exposure to these drugs.

Ormeloxifene inhibits tumorsphere formation in pancreatic stem cells
We observed a significant effect of ormeloxifene on tumorsphere formation in CSCs as reflected by a reduction in size and number of tumorspheres in cells upon treatment, suggesting the clonogenic depletion of the CSCs. In contrast, GDC-0449 did not show a significant effect on secondary tumorsphere formation (Supplementary Fig. S2D).

Ormeloxifene inhibits SHH signaling in PDAC cells
The SHH signaling pathway has been implicated in the development of pancreatic cancer (9). Therefore, PDAC cells were treated with ormeloxifene and changes in the SHH signaling pathway were evaluated by Western blot and qRT-PCR analyses. Ormeloxifene treatment effectively inhibited SHH expression at protein and mRNA levels at indicated concentrations (Fig. 2D and Supplementary Fig. S3A and S3B). Ormeloxifene treatment also inhibited the expression of Gli-1, SMO, cyclin D1, and p-AKT, the
key downstream proteins that drive the oncogenic signaling of SHH pathway in BxPC-3 and MiaPaca cells (Fig. 2D and Supplementary Fig. S3A; ref. 12). Ormeloxifene treatment also increased the expression of tumor-suppressor SUFU, which interacts directly with Gli-1 proteins to repress SHH signaling (Fig. 2D and Supplementary Fig. S3A; ref. 32).

Importantly, ormeloxifene treatment caused a marked (≥70%) decrease in the expression of the SHH transcription factor, NF-κB-65 (33), and its downstream target, cyclin D1 (34), within 24 hours (Fig. 2D and Supplementary Fig. S3C). Cyclin D1 is an important mediator of SHH-induced cell proliferation and carcinogenesis. These data suggest that ormeloxifene treatment effectively inhibits tumorigenic phenotypes via modulation of SHH and its downstream signaling molecules.

Ormeloxifene and gemcitabine in combination induce apoptosis in PDAC cells

We investigated whether ormeloxifene treatment enhanced the apoptotic index in gemcitabine-resistant PDAC cells (Panc-1 and BxPC-3). Our data show that when combined, ormeloxifene (15 μmol/L), and gemcitabine (100 nmol/L) induced a significantly higher (21%) apoptotic population in 24 hours as compared with...
ormeloxifene and gemcitabine treatment alone (Fig. 3A). However, PI-positive postapoptotic/necrotic cell population was relatively small, suggesting that the induced cytotoxicity was predominantly through activation of apoptotic pathways. These data suggest that ormeloxifene-alone induced cell death does not involve the release of phosphatidylserine onto the outer leaflet,

Figure 3.
Ormeloxifene in combination with gemcitabine induces apoptosis and modulates key targets of SHH pathway. A and B, flow cytometric analysis of Annexin V-positive cells and cells in G0–G1 stage after treatment. Bars represent mean ± SD; n = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001 as compared with CT. C, relative fold change in the mRNA levels of key molecules involved in SHH pathway by qRT-PCR. Bars represent mean ± SD; n = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 as compared with CT. D, Western blot analysis indicating the effect of ormeloxifene (15 μmol/L), gemcitabine (100 nmol/L), and their combination on the important proteins in SHH pathway. Data are representative of one of three similar experiments. ORM, ormeloxifene; GEM, gemcitabine.

ormeloxifene and gemcitabine treatment alone (Fig. 3A). However, PI-positive postapoptotic/necrotic cell population was relatively small, suggesting that the induced cytotoxicity was predominantly through activation of apoptotic pathways. These data suggest that ormeloxifene-alone induced cell death does not involve the release of phosphatidylserine onto the outer leaflet,
indicative of Annexin V–positive apoptotic cells and mitochondrial apoptotic signaling. Instead, it may involve death receptor–mediated extrinsic apoptotic signaling. Therefore, we sought to investigate the effect of ormeloxifene on cell-cycle phase distribution. Typically, D-type cyclins are required for the progression of cells from the G1-phase of the cell cycle to S-phase (35).

Ormeloxifene treatment decreased the expression of cyclin D in BxPC-3 and MiaPaca cells (Fig. 2D and Supplementary Fig. S3A). Ormeloxifene treatment led to cell-cycle arrest at sub-G0–G1 phase in Panc-1 and BxPC-3 cells. Cells in sub-G1-phase increased up to 74% after ormeloxifene treatment (15 μmol/L), while cells in the S-phase decreased from 19% to 5%. However, gemcitabine treatment did not show an additional effect on cell-cycle phases (Fig. 3B). Similar effects in cell-cycle phase distribution were observed in BxPC-3 cells, which showed significant inhibition of the G2-M phase upon treatment with ormeloxifene and gemcitabine in combination.

Ormeloxifene and gemcitabine combination targets SHH signaling pathway and inhibits CIM in PDAC cells

In addition, we investigated combinatorial effects of ormeloxifene and gemcitabine on SHH and downstream signaling molecules. Treatment with ormeloxifene and gemcitabine has relatively more pronounced inhibitory effects on the expression of SHH, Gli-1, and SMO as compared with ormeloxifene or gemcitabine alone (Fig. 3D). This reveals the potentiated effects of ormeloxifene in combination with gemcitabine. We also confirmed these results by qRT-PCR analysis and observed an apparent decrease in the mRNA levels of main effectors of the SHH signaling pathway in response to ormeloxifene alone or in combination with gemcitabine. This included decreased expression of SHH (4-fold), SMO (5-fold), and patched 1/2 (PITCH1/2) compared with the control (Fig. 3C). Ormeloxifene alone or in combination with gemcitabine also showed a marked (~40%) decrease in the level of antiapoptotic, Bcl-xl protein (Fig. 3D). The Bcl-xl protein is also an important mediator of SHH and is transcriptionally regulated by SHH through the Gli-1 transcription factor (34). In addition, ormeloxifene alone or in combination with gemcitabine inhibited the Gli-1 and NF-kB-B-65 transcriptional activity in PDAC cells (Fig. 4A and Supplementary Fig. S3C). These results present first evidence that ormeloxifene inhibits the SHH–Gli-1 signaling pathway in PDAC.

Moreover, we evaluated the ability of ormeloxifene and gemcitabine to inhibit tumor progression and found that ormeloxifene inhibited motility (Fig. 4B) and the migratory ability of PDAC cells as demonstrated by wound healing (Fig. 4C).

Ormeloxifene and gemcitabine combination efficiently abrogates TGFβ-induced SHH signaling

The interactions among the stromal and tumor cells and the various cytokines embedded in the ECM contribute to the neoplastic phenotype (36). In addition to the activated tumor-stromal myofibroblasts (characterized by the expression of contractile genes such as smooth-muscle actin, αSMA; ref. 37), the activated PSCs that are characterized by expression of the stellate cell activation-associated protein (cygb/STAP) are identified as the major source of the excessive stromal ECM production in pancreatic tumors (6). Here, we show for the first time that indirect coculture of PDAC cells with PSCs (stimulated with TGFβ) induced enhanced secretion of SHH and chemokine CXCL12 (stromal cell–derived factor-1, SDF1) that was abrogated by ormeloxifene alone or in combination with gemcitabine. Gemcitabine treatment alone did not show any effect as observed through ELISA of the conditioned media in which the PDAC cells were cultured (Fig. 4D). CXCL12 is abundantly produced by the stromal cells that induce SHH expression, which promotes progression, metastasis, and chemoresistance of PDAC cells (38).

In addition, treatment with ormeloxifene alone or in combination inhibited the proliferation of PSCs as depicted by the decreased expression of αSMA and cygb/STAP in the immunofluorescence of PSCs (Fig. 4D, bottom). These results suggest that ormeloxifene not only reduces the number of stromal cells involved in the development of pancreatic fibrosis but also inhibits the paracrine SHH signaling between cancer and stromal cells that leads to desmoplasia and causes chemoresistance.

Combined ormeloxifene and gemcitabine treatment effectively inhibits tumor burden in mice model

To investigate the anticancer effects of ormeloxifene, we used a subcutaneous (for solid tumor) and i.p. (metastatic) preclinical murine xenograft model generated with gemcitabine-resistant BxPC-3 cells. Both ormeloxifene and gemcitabine, administered alone, inhibited overall tumor burden, but combination treatment of the two was more efficacious than either of them alone (Fig. 5A and B). When compared with the control mice, mice treated with ormeloxifene (P = 0.0301) or gemcitabine (P = 0.0009) or ORM+GEM in combination (P < 0.0001) showed a marked reduction in tumor weight (Fig. 5B). Moreover, in the i.p. model, tumors barely developed in the ORM+GEM–treated group. Upon further examination, we also found there were fewer or no metastases in the mice treated with ormeloxifene alone or in combination with gemcitabine (Fig. 5B, inset table). miR-132 is downregulated in pancreatic cancer, which contributes to pancreatic cancer development (39). Treatment of ormeloxifene alone or in combination with gemcitabine leads to increased levels of miR-132 in xenograft tumors (Fig. 5C). These data further confirm that ormeloxifene treatment along with gemcitabine could be an effective therapeutic modality for pancreatic cancer.

Ormeloxifene inhibits tumor desmoplasia and the host cells invading the tumor

To elucidate the basis of the potentiated antitumorigenic effects of ormeloxifene in combination with gemcitabine in mice, we analyzed the FFPE tumor tissues through tumor histopathology, immunofluorescence, and IHC analyses. We observed a clear inhibition of Gli-1 expression in tumor tissues from mice treated with either ormeloxifene alone or in combination with gemcitabine (Fig. 5D). In contrast to vehicle- or gemcitabine-treated mice, which exhibited profuse desmoplastic tumor stroma, mice treated with ormeloxifene showed markedly depleted desmoplastic stroma. This was evidenced by a decrease in collagen I content in tumor xenografts and the invading host mice cells migrating into the tumors (Fig. 6A). It was found that only ormeloxifene, but not gemcitabine, reduced the amount of collagen I deposition. Interestingly, these differences were apparent in mice treated with ormeloxifene or ORM+GEM. In addition, ormeloxifene alone or in combination with gemcitabine treatment showed decreased number of activated stromal cell populations as identified by the reduced expression of αSMA and fibroblast surface protein (FSP)–positive stromal myofibroblasts (Fig. 6A) and cygb/STAP positive activated stellate cells (Fig. 6B; ref. 37). Gemcitabine alone treatment did not show any effects on these parameters. This decrease
in proliferation was accompanied by a decrease in SHH expression in ormeloxifene and ORM-+GEM–treated tumor tissues (Fig. 6B). Furthermore, we analyzed these tumor tissues for the presence of tumor-infiltrating macrophages and found a large increasing population of macrophages in ormeloxifene-treated mice tumor tissues (Fig. 6B) that might become tumoricidal and facilitate the depletion of the tumor stroma (40, 41). This signifies that ormeloxifene alone or in combination with gemcitabine inhibits the host cells invading the tumor tissue and disrupts the desmoplastic stroma that can facilitate the delivery and enhance the efficacy of gemcitabine.

Discussion
Pancreatic tumors are typically characterized by a high desmoplastic reaction (42). Desmoplasia plays an important role to initiate cross-talk between stromal-cancer cells, limit the delivery and effectiveness of chemotherapy, and induce chemoresistance. The SHH pathway is a major player for desmoplasia and is activated in both stromal and cancer cells in PDAC (7, 43). Therefore, suppression of the Hh pathway and desmoplasia may limit the molecular/clinical course of PDAC and improve drug(s) access in tumors (13). Currently available Hh pathway antagonists, including GDC-0449, have been investigated as a single agent or in combination with conventional chemotherapies for cancer treatment (14, 15). GDC-0449 is an SMO (Hh) inhibitor approved by the FDA for the treatment of locally advanced and metastatic basal cell carcinomas. But severe toxicity issues and adverse effects (fatigue, nausea, asthenia, mucositis, peripheral sensory neuropathy, dysgeusia, muscle spasms, and dehydration) and the lack of strong efficacy, limits its use in cancer therapy (44). In addition, no significant improvement in survival of pancreatic, colon, and ovarian cancer patients is noticed in recent clinical trials of Hh signaling inhibitors. As other signaling pathways...
such as PI3K or TGFβ signaling; refs. 45, 46) are also known to activate transcriptional activity of Gli in addition to SMO, the therapeutic efficacy of SMO inhibitors is compromised in cancer. This is a probable rationale for shifting interest from SMO inhibitors toward more specific Gli inhibitors in order to effectively suppress the Hh signaling pathway. In this endeavor, we have identified ormeloxifene, a nonsteroidal triphenylethylene compound that effectively blocks the Hh signaling pathway by inhibiting the important effectors of this pathway, such as SHH, SMO, Gli-1, and SDF-1 (CXCL12). Ormeloxifene disrupts multiple paracrine factors that are important for the maintenance of Hh signaling, and thus inhibits stromal and tumor cell cross-talk within the tumor.

Experimental investigations indicate that ormeloxifene inhibits proliferation, invasion, and clonogenicity of PDAC cells (Fig. 1 and Supplementary Fig. S2), comparable with cells treated with GDC-0449. In addition, reduced tumorsphere formation of CSCs that were treated with ormeloxifene indicates that ormeloxifene also inhibits pancreatic CSC proliferation and self-renewal. This suggests that the anticancer effects of ormeloxifene are greater or comparable with GDC-0449. Investigations of the mechanism of ormeloxifene-induced cell death showed the induction of cell-

**Figure 5.** Ormeloxifene and gemcitabine in combination inhibit tumor growth in pancreatic xenograft mice. A, photographs of xenograft mice from each treatment group. B, average tumor volume and average tumor weight was determined. Bars represent mean ± SD. * P < 0.01; ** P < 0.0001. The corresponding table shows the effect of ormeloxifene on tumor development and dissemination. C, in situ hybridization for tumor-suppressor miR-132 was performed on the excised tumor tissues from treated mice. D, IHC staining showing the inhibition of Gli-1 expression in tumor tissues from mice treated with ormeloxifene alone or with gemcitabine. Images were captured using Nikon phase contrast microscope with ×200 objective.

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It has been reported that NF-κB (33) and SHH (7, 43) signaling pathways play crucial roles in PDAC progression and drug resistance, including gemcitabine. Ormeloxifene treatment stabilizes IκBα, which inhibits protein and transcriptional activity of NF-κB-65, preventing it from binding to the SHH promoter and leading to its transcription (33). This was further confirmed on finding that ormeloxifene alone or in combination with gemcitabine inhibits the main downstream targets of SHH, SMO, and PTCH1/2 and downregulates the expression and transcriptional activity of Gli-1 in PDAC cells. No such effects were found in cells treated with gemcitabine alone. In the absence of SHH, cells have small amounts of PTCH1/2 and Gli and therefore, the high concentrations of these transcripts generally indicate involvement of the SHH pathway in PDAC (47). The aberrantly activated SHH binds to its receptor PTCH1/2 and inhibits the suppressive effect of PTCH1/2 on SMO, which activates Gli-1 to transcribe Hh oncogenic target genes (43). Ormeloxifene inhibits AKT phosphorylation, which is known to activate Gli-1 (48). The observations collected from coculturing the PDAC cells with stromal cells indicate the inhibition of paracrine stromal cell signaling through the inhibition of their proliferation and secretion of SHH and SDF1. All these results confirm that ormeloxifene inhibits Hh signaling in PDAC cells; thus, we hypothesized that it might also disrupt the stroma of pancreatic tumors and alter the desmoplastic reaction.

Recent studies implicate the profound role of stroma in drug resistance in numerous tumor models (49). Thus, targeting both neoplastic cells and stromal components are emerging for PDAC (50). The enhanced antitumor effect of ormeloxifene and gemcitabine combination treatment was observed in xenograft mouse models when compared with their treatment alone. An abundant stromal component was observed in the control and gemcitabine-treated tumor tissues while mice treated with ormeloxifene alone or ORM+GEM combination showed markedly less stromal component and invaded stromal tissue. This was indicated by the presence of reduced numbers of stroma myofibroblasts infiltrating the tumor tissue, as indicated by reduced myofibroblasts expressing αSMA and cygb/STAP expression in the tumor. ORM, ormeloxifene; GEM, gemcitabine.

Ormeloxifene Suppresses Desmoplasia in Pancreatic Cancer

 Altogether, the results indicated that ormeloxifene potentiates the anticancer effect of gemcitabine when used in combination.

Figure 6. Representative photomicrographs of immunofluorescence studies on excised xenograft tumor tissues using confocal microscopy. A and B, treatment of ormeloxifene alone or in combination with gemcitabine inhibited both human and mouse collagen I, FSP, and therefore reduced the number of total stroma cells within the tumor, which is indicated by reduced myofibroblasts expressing αSMA and cygb/STAP. This was observed using a laser scanning confocal microscope (Nikon TIRF), original magnifications ×200. In addition, tissues were stained for F4/80 that indicated increased number of macrophages infiltrating into the tumor. ORM, ormeloxifene; GEM, gemcitabine.

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cycle arrest at G0–G1 phase, suggesting that ormeloxifene may induce apoptosis. It was also an intriguing observation that treatment of ormeloxifene in combination with gemcitabine showed an increasing population of Annexin V–positive cells as compared with when both were used alone. These results indicate that in the presence of ormeloxifene, gemcitabine induces higher apoptotic cell death that might be triggered through the mitochondrial pathway. Alternatively, the other possibility is that ormeloxifene might involve death receptor–mediated cell death.
reduction of tumor progression, invasion, metastasis, and chemoresistance (Fig. 7). This facilitates the anticancer effects of ormeloxifene and potentiates the chemotherapeutic effects of gemcitabine for pancreatic cancer treatment. Our results have important implications toward the development of effective therapy for pancreatic cancer treatment.

**Conclusion**

In summary, our study provides new evidence regarding the anticancer effects of ormeloxifene in PDAC. This study demonstrates novel role of an existing drug, ormeloxifene to inhibit the SHH pathway and desmoplasia, resulting in tumor growth inhibition and potentiation of the antitumor effect of gemcitabine. This suggests that a combination of ormeloxifene and gemcitabine may have the capacity to inhibit the SHH signaling cascade in PDAC cells and alter the behavior of surrounding stromal cells so that cancer progression is repressed. Therefore, this study provides evidence that ormeloxifene in combination with gemcitabine could serve as a novel therapeutic intervention for pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**


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