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 (GEM). 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 (ORM), has potent anti-cancer properties and depletes tumor-associated stromal tissue by inhibiting the SHH signaling pathway in PDAC. We found that ORM inhibited cell proliferation and induced death in PDAC cells, which provoked us to investigate the combinatorial effects of ORM with GEM at the molecular level. ORM caused potent inhibition of the SHH signaling pathway via downregulation of SHH and its related important downstream targets such as Gli-1, SMO, PTCH1/2, NFκB, p-AKT and Cyclin D1. ORM potentiated the anti-tumorigenic effect of GEM by 75% in PDAC xenograft mice. Further, ORM 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 ORM in combination with GEM restored the tumor suppressor miR-132, and inhibited stromal cell infiltration into the tumor tissues. Additionally, invasiveness of tumor cells co-cultivated with TGFβ-stimulated human pancreatic stromal cells was effectively inhibited by ORM treatment alone or in combination with GEM. We propose that ORM has high therapeutic index and in a combination therapy with GEM 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 (GEM), extends patient survival by only a few months (1). Newer treatment options for PDAC patients are FOLFIRINOX and nab-paclitaxel/GEM, which improved overall survival by 4.3 and 1.8 months over GEM therapy, respectively (2, 3). However, safety profile of these drugs is less favorable than GEM 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 \textit{Kras} and/or inactivating mutations or loss of expression of tumor suppressor genes (including \textit{DPC4}, \textit{p16}, \textit{p53}, and \textit{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 is 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 (13). SHH is a member of the Hedgehog (Hh) family of secreted signaling proteins, having diverse functions during vertebrate development (10). 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 (11). Depletion of tumor stroma leads to the increasing functional vasculature that provides a
feasible avenue for efficient therapeutic drug delivery (12). Additionally, Hh signaling plays a key role in the maintenance of pancreatic cancer stem cells (CSCs) that are involved in drug resistance, cancer recurrence and poor clinical outcome (13). Therefore, molecular and/or chemical intervention to target Hh signaling and disrupt the microenvironment in tumors could be an interesting therapeutic approach for pancreatic cancer (12). Some of the well-known Hh signaling antagonists such as vismodegib (GDC-0449) have been investigated alone or as an adjuvant to the traditional anti-cancer 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 anti-cancer agents is a current active investigative approach. Ormeloxifene (ORM) is a non-hormonal, non-steroidal oral contraceptive molecule (18). Recent studies suggested that ORM may be effective in inhibiting breast cancer, head and neck cancer, and chronic myeloid leukemia cells (18). Moreover, ORM is reported to have an excellent therapeutic index and is safe for chronic administration (19). This study demonstrates the inhibitory role of ORM 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 to a known SMO inhibitor, GDC-0449, in PDAC cells. ORM disrupts the stroma of fibrotic pancreatic tumors and inhibits the proliferating stellate and myeloid cells involved in the development of pancreatic fibrosis. Further, the combinatorial effects of ORM with GEM induce increased GEM sensitivity. Additionally, these studies also suggest wide use of ORM 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 American Type Cell Culture collection (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). ORM was generously synthesized and provided by Fathi Halaweish, (South Dakota State University) as described earlier (23). GEM was purchased from Sigma Aldrich (catalog number G6423) and GDC-0449 from Sellekchem (catalog number S1082). Cells were treated with indicated doses of ORM, GEM and GDC-0449 after completely solubilized in ethanol, PBS and DMSO, respectively.

Cell proliferation by MTS assay

The anti-proliferative effect of ORM was determined after 48 hours using the CellTiter 96 AQeous One solution assay (catalog number G5421, Promega) using a microplate reader (BioMate 3 UV-Vis spectrophotometer, Thermo Electron Corporation). Ethanol- or PBS-containing medium served as the vehicle control. Additionally, the anti-proliferative effect of ORM 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 ORM.
The anti-proliferative 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 per well) were seeded in E-plate (Roche) following the xCELLigence Real Time Cell Analyzer (RTCA) DP instrument manual as provided by the manufacturer (Roche) (24). After 24 hours, ORM or the vehicle control was added and the experiment was allowed to run for 100 hours. Average baseline cell index for ORM treated cells compared to 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 x 10^6) were treated for 24 hours with ORM (15 µM) and GEM (100 nM) 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 ORM (15 µM) and GEM (100 nM) alone or in combination for 24 hours and stained with Telford Reagent containing propidium iodide (catalog number 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 NFκB transcriptional activity using a luciferase assay kit (catalog number E2940; Promega) according to the manufacturer's protocol. BxPC-3 and Panc-1 cells were transfected with luciferase reporter constructs (NFκB, gift from Dr. Ajay Singh, Mitchell Cancer Institute; Cignal GLI Reporter (luc) Kit, catalog number CCS-6030L, Qiagen) and treated with ORM and GEM alone or in combination for 24 hours. The normalized luciferase activity was expressed as a ratio of firefly luciferase to Renilla luciferase units.

Indirect co-culture of PDAC cells and pancreatic stromal cells

Human pancreatic stromal cell (PSC) fibroblasts and stellate cells were attained from an islet transplant program and maintained in CMRL-1066 medium (catalog number 15110, Corning) supplemented with 10% FBS, penicillin sodium and streptomycin sulfate at 37°C in humidified atmosphere containing 5% CO2. Human PSCs (3 x 10^6 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 x 10^6 cells/well), followed by treatment with ORM (10 μM) and GEM (100 nM) and incubated up to 2 days in DMEM medium. As previous studies have shown TGF-β to be a potent inducer of epithelial-mesenchymal transition (EMT) in several cancer cells including pancreatic cancer cells (26, 27), we used recombinant TGF-β (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 ORM for 12 days. The visible colonies (≥ 50 cells) were counted following hematoxylin staining (Fisher Scientific) and the percent of colonies was calculated as compared to 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 manufacturer's suggestions. After 48 hours incubation, the invading cells were stained and counted in 10 fields of view. Additionally, a wound healing migration assay was also used to evaluate the effect of ORM 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, $4 \times 10^4$ cells were seeded per chamber of CIM (cell invasion and migration) plate and the cells was analyzed in xCELLigence instrument at 37°C, 5% CO$_2$ for migration and invasion assays.

**Immunoblot analysis**
Human PDAC cells ($1 \times 10^6$) were treated with ORM (15 µM) and GEM (100 nM) 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 polymerase chain reaction (Q-RT-PCR)**

Total RNA was extracted using TRIZOL reagent (catalog number 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 Q-RT-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 $1 \times 10^3 / 100 \mu l$ well/96 well plate and treated with ORM or GDC-0449 (2.5-10 µM). 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 $1 \times 10^4 / 2 \ ml / 6$ 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 were purchased from Charles River Laboratories International, Inc., and maintained in a pathogen-free environment. The mice were
injected with BxPC-3 cells intraperitonally (i.p.) (3×10^6) and (5×10^6) cells in 200 μl PBS/matrigel subcutaneously. On day 15, the mice were treated with vehicle (ethanol), ORM (200 μg), GEM (500 μg), or their combination via intraperitoneal injections, thrice a week, for six 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}{4} 0.52 (l x w x h), as described previously (28) (Fig. S4). 45 days after the first drug injection, mice were euthanized and tumor burden (wet weight) and metastases were noted. The organs, including pancreas, were harvested and checked for metastases. The data were modeled with time (discrete), group (control, ORM, GEM and ORM+GEM), and the interaction between them. Primary analyses involved planned comparisons (separately for each time point) between control and ORM/GEM vs. ORM+GEM. Animal care was performed in accordance with institutional guidelines and all animal experiments were done using protocols approved by the Sanford Research Institutional Animal Care and Use Committee (IACUC).

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 employed an in situ hybridization technique and used a Biochain kit (catalog number 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.
Immunoflorescence and Immunohistochemical analyses

Immunoflorescence and Immunohistochemical 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 20X Apochromat objective for immunoflorescence. 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 Student's t test. Differences with P values of <0.05 are considered significant. Tumor size values were examined at the Day 50 point, using an analysis of variance approach. Tests of main effects (differences between treatments) and contrasts were performed.

Results

ORM treatment suppresses tumorigenic features of PDAC cells

ORM was found to have an anti-cancer effect on all tested PDAC cells (Fig. 1A and S1A). To confirm these results, we measured the growth in real time for 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 ORM significantly reduced the baseline cell index compared to the control cells (Fig. 1B). Further, ORM 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 ORM treatment (Fig. 1C). Moreover, ORM 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 (Fig. S1B) and cellular invasion by Matrigel invasion assay (Fig. S1C), which was further confirmed using the xCELLigence method (Fig. 2C).

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

ORM treatment inhibits tumorsphere formation in pancreatic stem cells

We observed a significant effect of ORM 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 (Fig. S2D).
ORM 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 ORM and changes in the SHH signaling pathway were evaluated by Western blot and qRT-PCR analyses. ORM treatment effectively inhibited SHH expression at protein and mRNA levels at indicated concentrations (Fig. 2D and S3A and B). ORM 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 signaling pathway in BxPC-3 and MiaPaca cells (11) (Fig. 2D and S3A). ORM treatment also increased the expression of tumor suppressor SUFU, which interacts directly with Gli-1 proteins to repress SHH signaling (32) (Fig. 2D and S3A).

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

ORM and GEM in combination induce apoptosis in PDAC cells

We investigated if ORM treatment enhanced the apoptotic index in GEM-resistant PDAC cells (Panc-1 and BxPC-3). Our data show that when combined, ORM (15 µM) and GEM (100 nM) induced a significantly higher (21%) apoptotic population in 24 hours as compared to ORM and GEM treatment alone (Fig. 3A). However, PI-positive post-apoptotic/necrotic cell population
was relatively small, suggesting that the induced cytotoxicity was predominantly through activation of apoptotic pathways. This data suggests that ORM-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 ORM 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). ORM treatment decreased the expression of cyclin D in BxPC-3 and MiaPaca cells (Fig. 2D and S3A). ORM 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 ORM treatment (15 µM), while cells in the S phase decreased from 19 to 5%. However, GEM 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 G2M phase upon treatment with ORM and GEM in combination.

**ORM and GEM combination targets SHH signaling pathway and inhibits cell invasion and migration in PDAC cells**

Additionally, we investigated combinatorial effects of ORM and GEM on SHH and downstream signaling molecules. Treatment with ORM and GEM has relatively more pronounced inhibitory effects on the expression of SHH, Gli-1 and SMO as compared to ORM or GEM alone (Fig. 3D). This reveals the potentiated effects of ORM in combination with GEM. 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 ORM alone or in combination with GEM. This included decreased expression of *SHH* (four-fold), *SMO* (five-fold) and *patched 1/2*
(PTCH1/2) compared to the control (Fig. 3C). ORM alone or in combination with GEM also showed a marked (~40%) decrease in the level of anti-apoptotic, 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). Additionally, ORM alone or in combination with GEM inhibited the Gli-1 and NFκB-65 transcriptional activity in PDAC cells (Fig. 4A and S3C). These results present first evidence that ORM inhibits the SHH–Gli-1 signaling pathway in PDAC.

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

**ORM and GEM combination efficiently abrogates TGF-β induced SHH signaling**

The interactions among the stromal and tumor cells and the various cytokines embedded in the extracellular matrix (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) (37), the activated pancreatic stellate cells (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 co-culture of PDAC cells with PSCs that are stimulated with TGF-β induce increased secretion of SHH and chemokine CXCL12 (stromal cell-derived factor-1, SDF1) was abrogated by ORM alone or in combination with GEM. GEM 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). Additionally, treatment with ORM alone or in combination inhibited the
proliferation of pancreatic stromal cells as depicted by the decreased expression of αSMA and
cygb/STAP in the immunofluorescence of PSCs (Fig. 4D; lower panel). These results suggest
that ORM 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 ORM and GEM treatment effectively inhibits tumor burden in mice model

To investigate the anti-cancer effects of ORM, we used a subcutaneous (for solid tumor) and
intraperitoneal (metastatic) pre-clinical murine xenograft model generated with GEM resistant
BxPC-3 cells. Both ORM and GEM, 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 to the control mice, mice treated with ORM (p = 0.0301) or GEM (p =
0.0009) or ORM+GEM in combination (p < 0.0001) showed a marked reduction in tumor weight
(Fig. 5B). Moreover, in the intraperitoneal 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 ORM alone or in combination with GEM (Fig. 5B, inset table). miR-132 is
downregulated in pancreatic cancer, which contributes to pancreatic cancer development (39).
Treatment of ORM alone or in combination with GEM leads to increased levels of miR-132 in
xenograft tumors (Fig. 5C). This data further confirms that ORM treatment along with GEM
could be an effective therapeutic modality for pancreatic cancer.
ORM inhibits tumor desmplasia and the host cells invading the tumor

To elucidate the basis of the potentiated anti-tumorigenic effects of ORM in combination with GEM in mice, we analyzed the FFPE tumor tissues through tumor histopathology, immunofluorescence (IF) and immunohistochemical (IHC) analyses. We observed a clear inhibition of Gli-1 expression in tumor tissues from mice treated with either ORM alone or in combination with GEM (Fig. 5D). In contrast to vehicle or GEM treated mice, which exhibited profuse desmoplastic tumor stroma, mice treated with ORM 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 ORM, but not GEM, reduced the amount of collagen I deposition. Interestingly, these differences were apparent in mice treated with ORM or ORM+GEM. Additionally, ORM alone or in combination with GEM 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) (37). GEM alone treatment did not show any effects on these parameters. This decrease in proliferation was accompanied by a decrease in SHH expression in ORM and ORM+GEM treated tumor tissues (Fig. 6B). Further, we analyzed these tumor tissues for the presence of tumor infiltrating macrophages and found a large increasing population of macrophages in ORM-treated mice tumor tissues (Fig. 6B) that might become tumoricidal and facilitate the depletion of the tumor stroma (40, 41). This signifies that ORM alone or in combination with GEM inhibits the host cells invading the tumor tissue and disrupts the desmoplastic stroma that can facilitate the delivery and enhance the efficacy of GEM.
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 Sonic hedgehog (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 (12). 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). Additionally, 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) (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 towards more specific Gli inhibitors in order to effectively suppress the Hh signaling pathway. In this endeavor, we have identified ORM, a non-steroidal 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). ORM 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 ORM inhibits proliferation, invasion and clonogenicity of PDAC cells (Fig. 1 and Fig. S2), comparable to cells treated with GDC-0449. Additionally, reduced tumorsphere formation of CSCs that were treated with ORM indicates that ORM also inhibits pancreatic CSC proliferation and self renewal. This suggests that the anticancer effects of ORM are greater or comparable to GDC-0449. Investigations of the mechanism of ORM-induced cell death showed the induction of cell cycle arrest at G0-G1 phase, suggesting that ORM may induce apoptosis. It was also an intriguing observation that treatment of ORM in combination with GEM showed an increasing population of Annexin V positive cells as compared to when both were used alone. These results indicate that in the presence of ORM, GEM induces higher apoptotic cell death that might be triggered through the mitochondrial pathway. Alternatively, the other possibility is that ORM might involve death receptor-mediated cell death. Altogether, the results indicated that ORM potentiates the anticancer effect of GEM when used in combination.

It has been reported that NFκB (33) and SHH (7, 43) signaling pathways play crucial roles in PDAC progression and drug resistance, including GEM. ORM treatment stabilizes IκB-α, which inhibits protein and transcriptional activity of NFκB-65, preventing it from binding to the SHH promoter and leading to its transcription (33). This was further confirmed on finding that ORM alone or in combination with GEM 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 when treated with GEM alone. In the absence of SHH, cells have small amounts of \textit{PTCH1/2} and \textit{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). ORM 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 ORM inhibits hedgehog 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 types (49). Thus, treatment paradigms targeting both neoplastic cells and stromal components are emerging for PDAC (50). The enhanced anti-tumor effect of ORM and GEM combination treatment was observed in xenograft mouse models when compared to their treatment alone. An abundant stromal component was observed in the control and GEM treated tumor tissues while mice treated with ORM 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 PSCs, αSMA, FSP and cygb/STAP expression in the tumor tissue. This might be well supported by the observations showing the inhibition of both mouse and human collagen I in tissues, as activated PSCs are the predominant source of collagen in the desmoplastic reaction in pancreatic cancers (6). Interestingly, we also observed an increased number of macrophages in the tumors obtained from ORM treated mice. The increasing macrophage recruitment to tumor site can be explained as the emergence of tumor immunity that serves as a part of immune surveillance for targeting tumor
stroma in the treatment of cancer. The activated macrophages may rapidly infiltrate the tumors, become tumoricidal and facilitate the depletion of tumor stroma.

These findings suggest that ORM inhibits desmoplastic reaction in PDAC. Due to the toxicity and morbidity of available drugs, there is an urgent need for effective therapies that could target both tumor and stromal compartments to regulate pancreatic tumor growth. Therefore, ORM might be a drug of choice as it is very safe for chronic use in humans. Our results signify that ORM is effective in targeting Hh and tumor progression signaling pathways. Our results emphasize that ORM interrupts the tumor-stromal interactions to inhibit the reciprocal relationship between these two components, leading to reduction of tumor progression, invasion, metastasis and chemoresistance (Fig. 7). This facilitates the anti-cancer effects of ORM and potentiates the chemotherapeutic effects of GEM for pancreatic cancer treatment. Our results have important implications towards the development of effective therapy for pancreatic cancer treatment.

**Conclusion**

In summary, our study provides new evidence regarding the anti-cancer effects of ORM in PDAC. This study demonstrates novel role of an existing drug, ORM to inhibit the SHH pathway and desmoplasia, resulting in tumor growth inhibition and potentiation of the anti-tumor effect of GEM. This suggests that a combination of ORM and GEM 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 ORM in combination with GEM could serve as a novel therapeutic intervention for pancreatic cancer.
Acknowledgements

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References


Legends

Figure 1. Determination of proliferation, clonogenicity and cytotoxicity profiles of ORM 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 MTS assay for 48 hours and is shown as percentage. (B) Clonogenicity assay was performed to determine the ability of cells to form colonies (percent inhibition) following treatment. Cells were photographed and counted using AlphaEaseFC™ (Alpha ImagerHP AIC) software analysis tool. Bars represent mean ± SD; n=3; *p<0.05 and **p<0.001.

Figure 2. ORM targets Sonic hedgehog signaling pathway and inhibits PDAC cell invasion and migration. Effect of ORM on (A) cell invasion through matrigel invasion assay (B) cell migration ability through migration assay. Cells were photographed and counted using an imaging system. Bars represent mean ± SD; n=3; *p<0.0001. (C) Effect of ORM on cell invasion...
and migration ability was confirmed using β-actin as an internal control. Flow cytometric analysis of Annexin V positive cells and cells in G0–G1 stage after treatment. Bars represent mean ± SD; n=3; *p<0.01, **p<0.001 and ***p<0.0001 as compared to CT. (C) Relative fold change in the mRNA levels of key molecules involved in Sonic hedgehog pathway by qRT-PCR. Bars represent mean ± SD; n=3; *p<0.01, **p<0.001 and ***p<0.0001. (D) Western blotting analysis indicating the effect of ORM and GEM on the important proteins in Sonic hedgehog pathway. Data are representative of one of three similar experiments.

**Figure 4. ORM and GEM combination inhibits Gli-1 transcriptional activity and inhibits pancreatic cancer invasiveness.** (A) Treatment of ORM alone or with GEM inhibited Gli-1 transcriptional activity and (B) inhibited cell migration. (C) Wound healing assay. The initial (0 hours) and the residual gap length, 48 hours after wounding, were analyzed from photomicrographs. (D) Indirect co-culture of PDAC and stromal cells and treatment with ORM alone and in combination with GEM. ELISA was performed to observe the effect on the secretion of key proteins (SHH and SDF1) involved in tumor stromal interactions. Bars represent mean ± SD; n=3; *p<0.01 and **p<0.001. Immunofluorescence indicates that treatment with ORM and GEM in the presence of pancreatic stromal cells (PSCs) reduces the number of myofibroblasts expressing Cygb/STAP and αSMA.

**Figure 5. ORM and GEM 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 and **p<0.0001. The corresponding inset table shows the effect of ORM 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) Immunohistochemical staining showing the
inhibition of Gli-1 expression in tumor tissues from mice treated with ORM alone or with GEM.
Bars represent mean ± SD; *p<0.01 and **p<0.001.

Figure 6. **Representative photomicrographs of immunofluorescence studies on excised xenograft tumor tissues using confocal microscopy.** (A and B) Treatment of ORM alone or in combination with GEM 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 20X. Additionally, tissues were stained for F4/80 that indicated increased number of macrophages infiltrating into the tumor.

Figure 7. **Diagrammatic representation of the ORM modulation of the Sonic hedgehog signaling pathway.**
Figure 1
### Figure 3

#### A

Comparative analysis of cell populations across different treatments for Panc-1 and BxPC-3 cells. The graphs show fold change in cell population for each treatment group.

#### B

Cell cycle analysis for Panc-1 and BxPC-3 cells under various treatments. The bar graphs indicate the percentage population in each phase (G0-G1, S, G2M).

<table>
<thead>
<tr>
<th>Cells: Panc-1</th>
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<td>Treatment</td>
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<td>ORM</td>
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<td>GEM</td>
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<table>
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<th>Cells: BxPC-3</th>
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<tr>
<td>Treatment</td>
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<td>CT</td>
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<tr>
<td>GEM</td>
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<tr>
<td>ORM+GEM</td>
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#### C

Relative fold change for SHH, SMO, PTCH1, and PTCH2 genes under different treatments for Panc-1 and BxPC-3 cells. The plots show statistical significance (indicated by asterisks).

#### D

Western blot analysis for SHH, Gli-1, Bcl-xL, and SMO proteins in BxPC-3 cells under various treatments. Beta-actin is used as a loading control.
**Figure 5**

**Effect of ORM on tumor development and dissemination**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Mean volume (mm³) of secondary tumors</th>
<th>Mean tumor weight (gm) (pr+sec)</th>
<th>No. of mice with dissemination to</th>
<th>Av. metastases</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>pancreas</td>
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<td>6</td>
<td>210.1</td>
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<td>1</td>
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<tr>
<td>ORM</td>
<td>6</td>
<td>9.2Φ</td>
<td>1.2</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>GEM</td>
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<td>55.9</td>
<td>0.6†</td>
<td>0*</td>
<td>0*</td>
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<tr>
<td>ORM + GEM</td>
<td>6</td>
<td>19.6Φ†</td>
<td>0.2*</td>
<td>0*</td>
<td>0*</td>
</tr>
</tbody>
</table>

*Significantly different from EtOH group (p<0.0001),  Φ Significantly different from EtOH group (p<0.01)
† Significantly different from EtOH group (p<0.001),  † Significantly different from GEM group (p<0.001)
‡ Significantly different from ORM group (p<0.01)
Figure 6
Ormeloxifene Suppresses Desmoplasia and Enhances Sensitivity of Gemcitabine in Pancreatic Cancer

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