Pluronic Polymer-Based Ormeloxifene Nanoformulations Induce Superior Anticancer Effects in Pancreatic Cancer Cells

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ABSTRACT: Utilization of safe cytotoxic agents with precise anticancer activity is considered as the prime focus of cancer therapeutics research. A greater incentive for such agents arises from the molecules/drugs that are already being used for other indications. Ormeloxifene (ORM) is a nonsteroidal, nonhormonal selective estrogen receptor modulator (SERM), which has been in human use for contraception purposes. Although in the recent past, many reports have suggested its emerging role as an anticancer agent, no significant attention was paid toward generating simple and safe nanoformulation(s) for improved therapeutic activity and tumor cell-specific delivery. Our aim is to develop nanoformulation(s) of ormeloxifene to improve its targeted delivery in tumor cells. We developed ormeloxifene nanoformulation(s) by utilizing various biocompatible polymers. The optimized formulations with pluronic polymers F127 and F68 show improved nanoparticle characteristics. These formulations show enhanced cellular uptake that allows ormeloxifene’s intracellular availability. We further evaluated its improved anticancer activity by performing cell proliferation, flow cytometry, and immunoblotting assays. Overall, this study confirms possible novel nanoformulation(s) of ormeloxifene to be evolved as a new therapeutic modality for cancer treatment.

INTRODUCTION

Pancreatic cancer (PanCa) remains the third leading cause of cancer-related deaths in the United States. Often pancreatic cancer is diagnosed at the locally advanced stage or when it has metastasized to distant sites due to nonspecific symptoms. Such circumstances are likely suitable for chemotherapy. Among many chemotherapies, 5-fluorouracil, gemcitabine, gemcitabine plus capcitabine (GemCap), Abraxane plus gemcitabine, and FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, and oxaliplatin or gemcitabine) are commonly administered to the patients with pancreatic cancer. These regimens are associated with numerous side effects such as nausea, vomiting, myelosuppression, hepatotoxicity, neurotoxicity, nephrotoxicity, and ototoxicity. In most cases, the heterogenic tendency of cancer progression not only promotes the aggressive nature of cancer cells but also facilitates resistance to chemotherapy. Repurposing of already existing drugs for a different indication has become a new attraction for researchers and is a clinically viable approach. The process of drug repurposing (drug reusing or repositioning) is a feasible and affordable mechanism to create newer therapeutic modalities as the safety and toxicity profiles of the drug(s) are already well-known. Ormeloxifene (ORM, C30H35NO3, a selective estrogen receptor modulatory molecule) was originally marketed as oral contraceptive pills in various Asian and African countries for humans. Recent literature demonstrates its anticancer activity against various cancers, such as cervical, ovarian, breast, head and neck, pancreatic, prostate, and chronic myeloid leukemia. A strong rationale to implement this molecule as an anticancer agent is based on a clinical study of ORM in 70 female patients, which suggests a 38.7% overall response rate with 6 months treatment. Additionally, ormeloxifene administration showed superior tumor growth inhibition in both rat and mouse models. These compelling evidence and excellent safety profile of ormeloxifene promote its clinical implications as an anticancer agent.
Producing various types of anticancer nanoparticle formulations can be a step closer toward achieving improved therapeutic benefits. Nanocarriers are utilized heavily for delivery of drugs also because they do not alter the loaded drug’s activities. A number of nanoparticle formulations that have been in clinic or under evaluation in clinical trials guide us toward achieving this newer path to treat cancer. Nanoparticles follow either passive or active targeting mechanisms to reach and accumulate in tumors, and thus, these can achieve improved therapeutic benefits. Therefore, we hypothesized that encapsulation of ormeloxifene in polymer matrices would increase its anticancer activity through increased cellular accumulation, escape from endosomal and lysosomal degradations, sustained release in the cytoplasm, and reduced exocytosis. Hence, in the present study, we report a simple paradigm for the generation of self-assembled polymer/pluronic-ormeloxifene nanoformulations (ORMNFs) by employing the solvent evaporation technique with two pluronic polymers to improve its therapeutic potential in pancreatic cancer cells. Owing to the miscible nature of this drug and polymer chain structure of the polymer and pluronic, ormeloxifene can be entrapped into polymer cores and delivered in a sustained manner for both in vitro and in vivo applications. These formulations were optimized for therapeutic applications based on particle size and morphology of particles and further characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), Fourier transform infrared (FT-IR), and circular dichroism (CD). It was apparent that formation of nanoparticles is visible within a few minutes to 60 min depending on the pluronic polymer employed. Ormeloxifene molecules selectively resided inside the core of polymer/pluronic micelles. We designed these formulations based on pluronic’s biocompatible nature. Our results suggest enhanced anticancer potential of pluronic-ormeloxifene nanoformulations in MiaPaCa and HPAF-II pancreatic cancer cell lines as demonstrated by decreased cellular proliferation and mito-chondrial membrane potential. This study will facilitate the preclinical development of ORMNFs as a novel modality for pancreatic cancer management.

RESULTS

Generation of ORMNFs and ORM’s Compatibility with Polymers. Pluronic ORM nanoformulations were prepared using the solvent evaporation method. We screened eight different pluronic polymers for ORMNF preparation and performed a TEM experiment for screening. Results from TEM images showed that only two pluronic polymers had shown uniform particle formation, and both F127-ORM and F68-ORM nanoformulations were spherical in shape with ~50 nm particle size (Figure 1). All formulations were compared to free ORM.

From this experiment, we selected F127-ORM and F68-ORM for further characterizations. To generate a successful and stable nanoformulation, it is necessary to validate the compatibility between the drug and polymer molecules. An optical microscopic experiment was performed to test ORM’s compatibility with F127 and F68 polymers. Images from different timepoints in this experiment confirmed that ORM was highly dispersed with these two polymers (Figure 2A). It was evident in this figure that free ORM formed aggregated clusters as time increased, whereas ORM with F127 and F68 was completely stable or compatible and fully miscible as no clumps or aggregates of the drug were seen in these images. Based on this, we propose a possible structure of nanoparticle formation with pluronic polymer and ORM (Figure 2B).

Physicochemical Characterization of ORMNFs. Particle size, surface charge, FT-IR, and CD spectra analyses were performed after each preparation to confirm the ORMNF production and the presence of ORM in nanoparticles. Results from DLS analyses revealed that ORMNF particle size and ζ-potential in 1× PBS aqueous condition (swollen nanoparticles) were around ~120 ± 1.7 nm and ~−10 ± 0.5 mV, respectively [(Figure 3A) size F127-ORM: 119 ± 1.6 nm, F68-ORM: 123 ± 1.8 nm and (Figure 3B) surface charge F127-ORM: −7 ± 0.3 mV, F68-ORM: −9 ± 0.7 mV]. The presence of ORM in ORMNFs showed characteristic peaks in the FT-IR spectral analysis (Figure 3C,D).

This suggests that ORM was well dispersed within F127 and F68 pluronic polymer matrices due to miscibility/compatibility. Such property offers superior encapsulation of ORM into the core of these two polymers. The analysis of conformational changes at the secondary structure of ORMNFs after their contact with bovine serum albumin (BSA) through CD spectra revealed that there were not any significant changes in the secondary structure of BSA–ORMNFs (Figure 4A–C). This set of data signifies less interaction with serum proteins and, thus, suggests longer half-life and superior adaptability of ORMNFs.

ORMNFs Inhibit Cellular Viability of Pancreatic Cancer Cells. Cancer cells proliferate in an uncontrolled manner, thus to qualify as an anticancer agent, a drug candidate should be able to inhibit cancer cell proliferation/growth. To examine the antiproliferative properties of ORMNFs, we exposed MiaPaCa and HPAF-II pancreatic cancer cell lines to drug treatments at different concentrations for 48 h for morphological changes and MTS assay. The results from phase-contrast images showed marked deteriorations in cellular morphology and viability of both MiaPaCa (Figure 5A) and HPAF-II (Figure 5B) cell lines in a dose-dependent manner. MTS data was in correlation with these findings as...
both cell lines (Figure 5C for MiaPaCa and Figure 5D for HPAF-II) exhibited significantly decreased cellular proliferation when compared to free ORM and vehicle controls.

ORMNFs Reduce Colony-Forming Ability of PanCa Cells. One of the major issues associated with cancer is that even a single cell is capable of growing into large colonies.32 Thus, this is extremely of high importance to examine ORMNFs’ potential to reduce the colony-forming ability of pancreatic cancer cells. Results from this experiment indicated that ORMNFs significantly inhibited the clonogenicity of MiaPaCa pancreatic cancer cell lines when compared to free ORM and vehicle controls (Figure 6A,B). However, F127-ORM seemed to have a greater inhibitory effect than F68-ORM in a dose-dependent manner.

ORMNFs Decrease Mitochondrial Membrane Potential. Depolarization of the mitochondrial membrane is an

Figure 2. (A) Optical microscopic images showing the compatibility of ORM with F127 and F68 polymers in ORMNFs. Dried drops of free ORM and ORMNF aqueous solution were microscopically examined. Free ORM shows excessive clumps of aggregation as time increases, whereas ORMNFs show uniform particle formation with no drug aggregation. Images were taken at 200×. (B) Schematic diagram showing the preparation steps of ORMNFs (F127-ORM and F68-ORM) by a solvent evaporation method.

Figure 3. Physicochemical characterization of ORMNFs. DLS measurements show (A) particle size and (B) surface charge (ζ-potential) of F127-ORM and F68-ORM nanoformulations. FT-IR spectra of (C) F127-ORM and (D) F68-ORM showing successful encapsulation of ORM in F127 and F68 polymer cores.
important and one of the early events in the initiation of apoptosis. Therefore, to investigate the ability of ORMNFs to initiate apoptosis, we performed a flow cytometry experiment. The tetramethylrhodamine (TMRE) staining

Figure 4. Circular dichroism spectra of ORMNFs. (A–C) Spectra showing secondary structure changes of ORMNFs after BSA interaction. ORM and its nanoformulations show stability and compatibility with bovine serum albumin protein as no major conformational shifts/changes were observed in CD spectra.

Figure 5. ORMNFs altered morphology of pancreatic cancer cell lines. (A) MiaPaCa and (B) HPAF-II cell lines were treated with ORMNFs and free ORM for 48 h and were imaged with a phase-contrast microscope at 200x after a careful visualization. Images clearly showed a decreased number of cells and apoptosis-like signs such as membrane blebbing and shrinkage. ORMNFs inhibited cell proliferation of pancreatic cancer cell lines. (C) MiaPaCa and (D) HPAF-II cell lines were treated with free ORM and ORMNFs at different concentrations for 48 h. Utilizing the MTS method, optical density was recorded at 490 nm to measure cellular proliferation. Results were normalized to free ORM and vehicle controls ETOH, F127, and F68. Error bars show standard error of the mean (SEM), n = 3. *p < 0.05.

Figure 6. ORMNFs reduced colony-forming ability of pancreatic cancer cells. (A) Images showing reduced clonogenicity of MiaPaCa cell lines. Cells were treated for 14 days with lower concentrations of ORM and ORMNF treatment. At the termination, cells were washed, fixed, stained, and imaged. (B) Graph bars show quantitation of these images. Colonies (~50 cells) were manually counted. Results were compared to free ORM and vehicle controls ETOH, F127, and F68. Error bars show SEM, n = 3. *p < 0.05.
method was used to detect the depolarization of the mitochondrial membrane. Our data from flow cytometry revealed that ORMNFs significantly reduced the mitochondrial membrane potential at the highest concentration of 30 μM when compared to free ORM in both MiaPaCa (Figure 7A) and HPAF-II (Figure 7B) cell lines.

ORMNFs Regulate Expression of Key Apoptotic Molecules. It was imperative to further confirm the apoptotic events at the molecular levels. To do this, we examined the expression level of two key players in apoptosis, namely, poly[ADP-ribose] polymerase (PARP) and B-cell lymphoma-extra-large (Bcl-xl) by utilizing immunoblotting. During apoptosis, total PARP undergoes degradation, and, thus, it increases the amount of cleaved PARP. Bcl-xl is an antiapoptotic marker, which promotes cell growth/survival.33,34 Figure 8A clearly illustrates that when MiaPaCa cells were treated with ORMNFs mainly with a higher concentration of 30 μM, apoptosis was induced as increased expression of cleaved PARP and decreased expression of Bcl-xl were observed when compared to free ORM and the control group.

ORMNFs Induce Ultrastructural Morphological Alterations. TEM imaging technique was utilized to evident the morphological changes and signs of apoptosis ultrastructurally in MiaPaCa cell lines. After 48 h of ORMNF (F127-ORM and F68-ORM) and free ORM exposure at 30 μM concentration, clear signs of apoptosis were evident. TEM micrographs revealed that after 6 h of drug treatment, cells did not show any obvious morphological changes as the nucleus and cell membrane were intact, whereas at later hours (48 h), cells were presented with ultrastructural changes as distorted nucleus and apoptotic vacuoles (endosomal and lysosomal) were observed (Figure 8B). These vacuoles are formed when the mitochondrial membrane depolarizes, and cells undergo apoptosis.33 Depolarization in the membrane of the mitochondria disintegrates the membrane wall, and, thus, it enhances the permeability and swelling of the organelle.33 ORMNF-treated cells showed more prominent apoptotic structures than free ORM and control cells. This set of data clearly indicated that ORMNFs were successfully delivered to pancreatic cancer cells, which further increased the therapeutic potential of ORMNFs in these cells.

■ DISCUSSION

Pancreatic cancer is a devastating disease with a poor 5 year survival rate of only ∼8%.3 The treatment for pancreatic cancer requires cytoreductive surgery with possible chemotherapy.37 Although chemotherapy is initially responsive, these tumors,
unfortunately, relapse due to the drug resistance.\textsuperscript{10} Thus, identifying new therapeutic molecules are urgently required to treat this deadly cancer. Enormous literature supports that nanocarrier(s) showed promising potential for the delivery of anticancer drugs.\textsuperscript{28,38,39} Additionally, nanoparticle-based anticancer drug formulations provide significant advantages over conventional small-molecule drug(s) by minimizing non-specific toxicity and enhancing therapeutic efficacy at tumors, and this is feasible due to their large surface-to-volume ratio.\textsuperscript{40−42}

Oremoxifene is a proven clinical oral contraceptive agent for humans and approved in India.\textsuperscript{24} In the recent past, considerable work has been focused to examine the mechanistic role of oremoxifene in cancer, but not many strategies exist for efficient delivery of oremoxifene to cancer cells. Earlier studies by Gupta and Jabrail\textsuperscript{43,44} have shown that physically cross-linked microspheres of chitosan with different molecular weights and degree of deacetylation offer sustained release of oremoxifene. Our recent investigations are the first examples of nanoparticle formulation of oremoxifene (poly-lactic-co-glycolic acid)-based nanoparticles), which demonstrated excellent tumor growth reduction.\textsuperscript{45,46} Previous efforts from our group have demonstrated that improved therapeutic effects of curcumin (an anticancer drug) were achieved using β-cyclodextrin and poly(β-cyclodextrin) inclusion complex, polymer nanoparticles, nanogels, and magnetic nanoparticle drug delivery vehicles.\textsuperscript{47−48} Polymeric micelles have also shown significance as a drug carrier for superior therapeutic potential.\textsuperscript{49−51} In the process of preparing these polymeric micelles, spontaneous formation of drug nanoparticle aggregation occurs by simply mixing the drug molecules and polymer micelles together, this results in a core−shell structure of the drug nanoparticle aggregates, and it is the main advantage over other nanocarriers.\textsuperscript{52,53} A cremophor EL free, paclitaxel-encapsulated poly(ethylene glycol)-b-poly(ε-caprolactone) (PEG-PLA) polymer micelle formulation under a trade name Genexol-PM has been approved by the Food and Drug Administration (FDA) for treating breast cancer.\textsuperscript{54,55} Another polymeric micelle based on Pluronics L61/F127-mixed micelles (SP1049C from Supratech Pharma Inc.) has successfully completed Phase II clinical trial for doxorubicin delivery to esophageal and gastroesophageal cancers.\textsuperscript{56} Selection of the type of block copolymers or pluronic polymers is a critical task, and it depends on the type of the drug that needs to be formulated. Pluronic polymers, mostly composed of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), have widely been studied to load various anticancer agents.\textsuperscript{53,57} These polymers have also been demonstrated to act as inhibitors of P-gp, which can sensitize tumor cells for therapeutic agents.\textsuperscript{58} In this context, developing a systemic formulation of oremoxifene would be highly valuable to overcome conventional systemic barriers and to facilitate intracellular drug accumulation at the tumor site.

In this current investigation, oremoxifene-encapsulated polymer micelle nanoformulations (ORMNFs) were successfully designed, developed, and tested for superior anticancer activity in MiaPaCa and HPAF-II pancreatic cancer cell lines. We employed various analytical tools to characterize ORMNFs and examined their anticancer activity using the MTS technique for cell proliferation, flow cytometry for mitochondrial membrane depolarization, and immunoblotting for protein expression. DLS data of ORM-encapsulated nanoformulations exhibited a particle size of ~120 nm and surface charge of ~−10 mV. Transmission electron microscopy images of ORMNFs indicated a smooth surface morphology for F68-ORM, whereas F127-ORM demonstrated an aggregative pattern of drug molecules and particle size of ~50 nm for both nanoformulations. CD spectra and optical images showed that ORMNFs are highly compatible and stable with serum albumin and within the formulation itself. The cytotoxic effects of free ORM and ORM nanoformulations exhibited a dose-dependent effect in decreased cell proliferation for both pancreatic cancer cell lines and in reduced colony-formation ability for MiaPaCa pancreatic cancer cell lines. At all treatment concentrations, ORM nanoformulations showed higher toxicity than free ORM. This enhanced cytotoxicity can be attributed to greater uptake of nanoformulations by the cells, which is a widely explained phenomenon. Additionally, ORMNFs induced apoptosis in these pancreatic cancer cell lines as evident by a decreased mitochondrial membrane potential, and the altered expression levels of PARP and Bcl-xl, two important apoptotic markers. Moreover, ORMNFs caused obvious ultrastructural changes in these cells as vacuole formations were observed with TEM, which further confirmed the induction of apoptosis.

Remarkably, an enhanced anticancer potential of ORMNFs indicates the feasibility for developing these novel nanoparticle drug formulations as a lead therapeutic modality for pancreatic cancer, provided that, additional studies are warranted for its preclinical and clinical investigations. Additionally, since oremoxifene has already been in human use for oral delivery, this novel formulation can be delivered orally as well as intravenously.

\section*{MATERIAL AND METHODS}

\textbf{Chemicals and Cell Culture.} All chemicals and reagents used in this work were purchased from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise mentioned. MiaPaCa and HPAF-II pancreatic cancer cell lines were purchased from ATCC (American Type Culture Collection) (Manassas, VA). These pancreatic cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and DMEM-F12 (Gibco, Gibco Laboratories, Gaithersburg, MD), respectively, with supplements: 4500 mg/L glucose, 4.00 mM l-glutamine, 10% heat-inactivated FBS (Atlantic Biologicals, Lawrenceville, GA), and 5 mL of 1× antibiotic/antimycotic (Sigma, St. Louis, MO). Cell lines were cultured at 37 °C under a humidified atmosphere of 5% CO₂. All of the cell lines used in this study were authenticated and checked for mycoplasma and other infections.

\textbf{Generation of ORM Nanoformulations (ORMNFs).} Eight different pluronic polymers, poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol), MW 1100 (L31), 1900 (L35), 2000 (L61), 2900 (L64), 4400 (L121), 5800 (P123), 8400 (F68), and 12 400 (F127), were screened to prepare ORM nanoformulations following a solvent evaporation technique\textsuperscript{59} with some modifications. Briefly, 10 mg of ORM in 1 mL of ethanol (ETOH) and 20 mg of the polymer in 1 mL of water were dissolved separately. Next, 100 μL of ORM solution was added dropwise to 900 μL of the polymeric solution, and it is then mixed with 1 mL of water under magnetic stirring at 800 rpm for about a minute in a 2 mL glass vial. Ethanol was evaporated under stirring overnight at room temperature to obtain a homogenous ORM nanoparticle formulation. This homogeneous suspension was filtered through a 0.45 μm syringe filter (Millex-LG, Millipore)
Compatibility of ORMNFs. Before testing these newly formed formulations in vitro and in vivo, it was imperative to evaluate the stability/compatibility between drug and polymer molecules. To determine the stability/compatibility of ORMNFs, we prepared 500 μg/mL aqueous solutions of ORMNFs, free ORM, and performed a visual evaluation of nanoparticle formation using the optical microscopy analysis. For this experiment, 2–3 drops of these freshly prepared solutions were placed onto a glass slide and were air-dried in a fume hood overnight. Slides were protected from exposure to dust and light, and the next morning, slides were imaged using optical microscopy (Olympus BX 41 microscope; Olympus, Center Valley, PA) at 200X.

Physicochemical Characterization. ORMNFs were characterized by various techniques including transmission electron microscopy (TEM), dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FT-IR), and circular dichroism (CD).

Transmission Electron Microscopy. The particle size and morphology of ORMNFs were examined using a JEOL-1210 transmission electron microscope (JEOL Ltd., Tokyo, Japan). For this study, a drop of ORMNFs (1 mg/mL suspension in water) was dispensed on a thin film of amorphous carbon deposited on a 200 mesh formvar-coated copper TEM grid (grid size: 97 μm) (Ted Pella, Inc., Redding, CA), followed by (2% w/v of uranyl acetate) staining solution, and air-dried. These nanoparticles on the TEM grid were viewed and imaged under TEM operating at 60 kV.

Dynamic Light Scattering. The particle size distribution and surface charge (zeta potential, ζ) of prepared ORMNFs were measured by the laser diffraction method using a Delsa Nano C particle size analyzer (Beckman Coulter, Brea, CA). After 2 min equilibration of the instrument, all of the measurements were performed at 25 °C. For measurements, ORMNFs (1 mg/mL suspension) were ultrasonicated for 30 s and transferred into a four-sided, clear plastic cuvette or a capillary ζ-potential cell for size distribution and ζ-potential. All results were the mean of 3 test runs.

Fourier Transform Infrared. To confirm ormeloxifene’s successful assembly in the pluronic polymer matrix, the FT-IR spectrometry experiments were performed. Using a Perkin-Elmer Spectrum BX spectrophotometer (Perkin-Elmer Inc., Norwalk, CT), FT-IR spectra were obtained for ORM and ORMNFs. For each FT-IR sample acquisition, 32 scans at the speed of 2 cm⁻¹ were recorded, and an average of these scans was presented as the FT-IR spectrum. The spectral range was chosen from 4000 to 650 cm⁻¹.

Circular Dichroism. A Jasco 815 circular dichroism (CD) spectrometer (JASCO International Inc., Ltd., Japan) was utilized to measure the changes in the secondary structure of the protein (bovine serum albumin, BSA) upon their interaction with ORMNFs. For this experiment, we used various concentrations of free ORM and ORMNFs (10–40 μM) with 10 μM of bovine serum albumin (BSA). The CD spectra of BSA or BSA-ORM or BSA–ORMNFs were recorded from 200 nm to 260 nm in a 1 cm quartz cell at 25 °C.

Cell Viability Assay. To perform cell proliferation/viability assay, 2 pancreatic cancer cell lines MiaPaCa and HPF-II were seeded at 5 × 10⁴ cells/well density in 96-well plates and allowed to adhere overnight. The next day, cells were treated with 10, 15, 20, 25, 30, and 40 μM concentrations of either free ORM or ORM nanoformulations (ORMNFs) for 48 h. ETOH and blank pluronic were used as vehicle controls for ORM and ORM nanoformulations, respectively. After 48 h, cells were washed twice with phosphate-buffered saline (PBS) and microscopically visualized for imaging. Images were captured with an Olympus microscope (Olympus, Center Valley, PA). Further, 25 μL of MTS reagent (CellTiter 96 AQueous, Promega Corporation, Madison, WI) was added to each well, and plates were incubated for the next 2–3 h. Post-incubation, absorbance was recorded at 490 nm spectrophotometrically using a BioMate 3 microplate reader (Thermo Fisher Scientific, Pittsburgh, PA). Each experiment was done in replicates of 6 and repeated 3 individual times.

Colony-Formation Assay. We utilized MiaPaCa cell lines for further long-term treatment evaluation of ORMNFs. At the density of 500 cells/well in 6-well plates, cells were plated and allowed to attach. When cells were fully attached usually in 24–30 h, different concentrations of ORMNFs were given to the cells and allowed to further incubate for 14–15 days. On the day of termination, plates were washed with PBS, fixed with ice-cold methanol (MEOH), stained with hematoxylin, and again washed with running water. Plates were then air-dried and imaged with a gel doc (BioRad, Hercules, CA). Colonies were manually counted, and each experiment was performed three individual times.

Mitochondrial Membrane Potential (ΔΨM) using TMRE. 2 × 10⁵ MiaPaCa and HPF-II cells were plated per well in 6-well plates and allowed to adhere overnight. The next day, cells were treated with ORM and ORMNFs at 10, 20, and 30 μM concentrations for 24 h. After 24 h, cells were washed with PBS, trypsinized, and centrifuged at 1000 rpm for 5 min to obtain the final pellet. The pellet was then resuspended in 1 mL PBS with 50 nM tetramethylrhodamine(TMRE, ethyl ester) stain containing in it, and cells were further incubated for 25–30 min in the dark at 37 °C. After the indicated time, cells were analyzed under the FL2 channel using a BD Accuri C6 flow cytometer. The experiment was performed three independent times.

Immunoblotting. For immunoblotting, 5 × 10⁶ MiaPaCa cells were plated in a 100 mm dish and allowed to adhere overnight. The next morning, cells were treated with ORM and ORMNFs at 20 and 30 μM concentrations for the next 48 h. Next, cells were washed with PBS, and lysates were collected by scraping cells with SDS buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and sonicated for 30–40 s during freeze–thaw cycles. The SYPRO Orange (Invitrogen, Carlsbad, CA) method was employed further to quantify the protein concentration. 50 μg of protein from each sample was loaded and separated by 4–20% SDS-PAGE gel electrophoresis at 150 V for 60 min. Proteins were then transferred onto a poly(vinylidene difluoride) (PVDF) membrane at 100 V for 90 min. The membranes/blots were next blocked with 5% skimmed milk in tris buffered Saline with Twin 20 (TBST) at room temperature for 1 h and further incubated with primary antibodies specific for poly[ADP-ribose] polymerase (PARP), B-cell lymphoma-extra-large (Bcl-xl), and β-actin at 4 °C for overnight. The next day, blots were washed thrice with TBST and incubated with goat antirabbit/mouse secondary antibodies for 1 h at room temperature. For protein signal detection, blots were washed again thrice with TBST for 15 min per wash and soaked in with a Lumi Light reagent (Roche, Nutley, NJ) and imaged with a gel doc (BioRad, Hercules, CA).

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Ultrastructure Morphological Analysis with TEM. TEM has become a powerful tool to study the morphological changes at the ultrastructural level and detect apoptosis in cells.\(^5\) To understand the ORMNF-induced apoptosis ultrastructurally, \(1 \times 10^6\) MiaPaCa cells per 100 mm dish were plated and allowed to adhere overnight. The next day, cells were exposed to 30 \(\mu\)M of ORMNFs or equivalent free ORM for 6 and 48 h. At the indicated timepoints, cells were washed with PBS, trypsinized, centrifuged, and fixed with 4% glutaraldehyde \((v/v)\) in 0.1 M cacodylate buffer and followed by 1% OsO\(_4\) solution fixation. Further, cells were washed with cacodylate buffer, and a graded series of acetone from 35 to 100% (35, 50, 75, 95, and 100%) was used to dehydrate the cell pellets. Next, cell pellets were embedded with low viscosity Spurr resin. Ultrathin sections of 70–90 nm thickness were cut/sectioned from these resin blocks utilizing an ultramicrotome. Cut sections were then transferred into 97 \(\mu\)m sized TEM grids (Ted Pella Inc., Redding, CA) and stained with uranyl acetate and lead acetate. After staining, these sections were visualized with transmission electron microscopy for ultrastructural changes.

Statistical Analysis. All statistical calculations were performed using Prism 6.0 (GraphPad Software Inc., San Diego, CA). The data are expressed as mean \pm standard error of the mean (SEM). Unpaired Student’s \(t\)-test was used for comparison of two groups. Differences were considered statistically significant when the \(p\)-value was <0.05.

CONCLUSIONS
To conclude, this work provides a proof-of-concept foundation that pluronic polymers can be a successful delivery vehicle for ormeloxifene. ORMNFs had a mean particle size of \(~120\) nm and \(\zeta\)-potential of \(~−10\) mV. These values are comparable with existing FDA-approved nanof ormulations for cancer therapeutics.\(^6\) Furthermore, these formulations exhibited superior anticancer activities against pancreatic cancer cell lines than free ormeloxifene. ORM nanof ormulations induced apoptosis through decreasing the mitochondrial membrane potential and altering the expression of apoptosis-related key proteins and the ultrastructure of these cells. Based on these results, we believe that ORM nanof ormulations could be a promising treatment regimen for pancreatic cancer. These results are warranted to further examine the anticancer efficacy of ORM nanof ormulations in clinically relevant mouse models.

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Notes
The authors declare no competing financial interest.

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