PEGylated Nanoparticles Loaded with 2-Methoxyestradiol for the Treatment of Uterine Leiomyoma in a Patient-Derived Xenograft Mouse Model

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Research Article

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Abstract

Leiomyomas, the most common benign neoplasms of the female reproductive tract, currently have limited medical treatment options. Drugs targeting estrogen/progesterone signaling are used, but side effects and limited efficacy in many cases are major limitation of their clinical use. Previous studies from our laboratory and others demonstrated that 2-methoxyestradiol (2-ME) is promising treatment for uterine fibroids. However, its poor bioavailability and rapid degradation hinder its development for clinical use. The objective of this study is to evaluate the in vivo effect of biodegradable and biocompatible 2-ME-loaded PEGylated nanoparticles in a patient-derived leiomyoma xenograft mouse model. Diblock copolymer nanoparticles loaded with 2-ME were prepared by nanoprecipitation. Female 6-week age immunodeficient NOG (NOD/Shi-scid/IL-2Rγnull) mice were used. Estrogen-progesterone pellets were implanted subcutaneously. Five days later, patient-derived human fibroid tumors were xenografted bilaterally subcutaneously. Engrafted mice were treated with 2-ME-loaded or blank (control) PEGylated nanoparticles. Nanoparticles were injected intraperitoneally and after 28 days of treatment, tumor volume was measured by caliper following hair removal, and tumor were removed and weighed. Up to 99.1% encapsulation efficiency was achieved, and the in vitro release profile showed minimal burst release, thus confirming the high encapsulation efficiency. In vivo administration of the 2-ME-loaded nanoparticles led to 51% growth inhibition of xenografted tumors compared to controls (P < 0.01). Thus, 2-ME-loaded nanoparticles may represent a novel approach for the treatment of uterine fibroids.

Introduction

The female reproductive system undergoes multiple cycles of growth and serial involution of myometrial smooth muscles cells. These cycles may predispose the cells to the development of mutations, potentially leading to the formation of a predominant uterine leiomyoma or a cluster of several leiomyomas also known as fibroids [1, 2]. Although benign in nature, the presence of leiomyomas can cause heavy uterine bleeding that lasts longer than normal menstruation, chronic iron deficiency, progressive pelvic pressure and pelvic pain, improper implantation of the embryo, pregnancy loss, failure of labor to progress, and may mask malignant tumors [2]. Uterine leiomyomas are the most common gynecologic tumors in women and the most common indications for hysterectomy [3]. It is estimated that nearly 80% of Black women and 70% of white women will have at least one leiomyoma by age 50 [4, 5].

Estrogen and progesterone signaling have been strongly implicated in fibroid growth [6, 7]. Thus, current medical treatment strategies for fibroids largely target the estrogen and progesterone systems, including combined estrogen/progestin contraceptive pills, progestins, gonadotropin-releasing hormone (GnRH) analogues, GnRH antagonists, selective progesterone receptor modulators (SPRMs), and aromatase inhibitors [8, 9].

Leiomyomas are characterized by cellular proliferation and excessive deposition of disordered extracellular matrix. Thus, an antiproliferative drug could suppress tumor growth and the synthesis of the extracellular matrix [10]. 2-methoxyestradiol (2-ME) is a naturally occurring estrogen metabolite found at
low levels in human plasma. This endogenous metabolite has shown effective antiproliferative and antiangiogenic effects in several in vitro and in vivo studies [11]. Ding et al. reported that 2-ME was able to induce apoptosis via mRNA and phosphorylation of Bcl-2 in human ovarian cells [12]. In vitro studies in several cell types such as uterine leiomyoma cell lines (ELT3, SK-LMS, and huLM), esophageal cell lines (WHCO3), nasopharyngeal cell lines (CNE2) and carcinoma cells have shown promising results, including inhibited collagen synthesis [13–19]. Clinical studies including Phase I and Phase II clinical trials have shown that oral administration of 2-ME was tolerated with patients and had anticancer effects [20–21].

However, the oral bioavailability of 2-ME is very low (1 to 2%) due to poor water solubility, extensive glucuronidation, and high plasma protein binding (97.4%) [22]. Patients receive high oral doses up to 3,000 mg twice a day to achieve modest plasma concentrations. Even with this high oral dose of 2-ME, the plasma concentration remains in the nanograms per mL range. In addition, 2-ME has a half-life of 1 to 2 days, suggesting extensive enterohepatic recycling [20–24]. These pharmacodynamic disadvantages overshadow 2-ME’s practical use as a therapeutic agent [10–16]. Therefore, in order to overcome the low solubility and poor bioavailability of 2-ME and still maintain its antiproliferative effects, Ali et al. [10] developed a novel therapeutic approach using biodegradable biocompatible nanoparticles encapsulating 2-ME. Several nanoparticle formulations were tested and were shown to have significantly higher cytotoxicity in a uterine leiomyoma cell line (huLM) in comparison to conventional 2-ME alone. Amongst the different types of nanoparticles investigated, poly(lactic-co-glycolic acid) (PLGA) nanoparticles demonstrated the most promising effect in the huLM cells [10].

Biodegradable polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers (PLGA) have received great interest because of their high degree of crystallinity and hydrophobicity as well as their potential for controlled drug release [24]. The biocompatible polymer poly(ethylene glycol) (PEG) can prolong the circulation time of nanoparticles. Due to the hydrophilic nature of PEG, PEGylated nanoparticles possess a stealth property which reduces phagocytic uptake [25, 26]. The aim of the current investigation was to expand upon the work of Ali et al. by preparing PEGylated PLGA nanoparticles loaded with 2-ME. The utility of these PEGylated nanoparticles to treat fibroids was tested in vitro as well as in an in vivo immunodeficient leiomyoma xenograft mouse model. Treatment with the 2-ME-loaded PEGylated PLGA nanoparticles led to a significant reduction in fibroid volume. Histological evaluations were performed to assess the expression of Cyclin D1, Ki-67, progesterone receptor, and estrogen receptor in the tumors.

**Materials And Methods**

**Nanoparticle preparation**

Diblock PEGylated poly(lactide-co-glycolide) (PEG-PLGA, RGPd5055) was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). 2-methoxyestradiol (2-ME) was obtained from Sigma (St. Louis, MO). Tetrahydrofuran (THF) was obtained from Fisher Scientific (Fair Lawn, NJ). 2-ME-loaded
PEG-PLGA nanoparticles were prepared using a modified solvent displacement/nanoprecipitation technique [27]. Briefly, a solution containing PEG-PLGA and 2-ME in THF was mixed for 3 hours at room temperature. This solution was injected at a flow rate of 6 mL/min with a variable-speed peristaltic pump (Traceable Calibration Control Company, Friendswood, TX) into 5 mL of water stirring at 500 rpm. After the injection, the nanosuspension was left overnight while stirring at 900 rpm for complete evaporation of the THF. The following day, the nanosuspension was stored at 4°C until further use.

**Nanoparticle size and zeta potential**

Z-average particle size and polydispersity index were determined using dynamic light scattering with a high-performance particle sizer (HPSS, Malvern Instruments, Malvern, UK). Nanoparticles were diluted in distilled water and each set of 10 sample runs at 25°C was repeated three times. The zeta potential (ζ) was determined in triplicate using a Zetasizer 2000 (Malvern Instruments, Malvern, UK).

**Analytical method**

Drug concentrations for determining encapsulation efficiency and drug release were quantified by high performance liquid chromatography (HPLC). A C18 Symmetry® analytical column (4.6 × 75 mm, Waters, Milford, MA) was used for the analysis. An isocratic elution of 1 mL/min was performed using a Waters® 1525 binary pump. The mobile phase consisted of acetonitrile:0.5% formic acid (65:35, v/v) and each injection ran for 30 minutes. The injection volume was 10 µL. Samples were spiked with an internal standard (letrozole, 10 µg/mL) and injections were performed using a Waters® 2707 autosampler. Detection was carried out using a Waters 2475 fluorescence detector at an excitation wavelength of 285 nm and an emission wavelength of 325 nm. The retention times of 2-ME and letrozole were found to be 20.85 ± 0.02 and 9.72 ± 0.11 minutes, respectively. Correlation coefficients (R²) of 0.999 and 0.998 were obtained over a linear concentration range of 50-115 µg/mL and 0.12-115 µg/mL for 2-ME and letrozole, respectively. The limits of detection were 5.7 ng/mL for 2-ME and 10 ng/mL for the internal standard letrozole.

**Drug encapsulation and drug release**

To determine the total amount of drug encapsulated within the nanoparticles, an ultracentrifugation technique was used to separate free (unencapsulated) drug from the drug contained within the nanoparticles. Briefly, 1 mL of nanoparticle suspension was added to 5 mL of purified water. This mixture was centrifuged at 55,000 × g to create a pellet containing nanoparticles. The supernatant containing free drug was carefully collected and the unencapsulated drug concentration was quantified using the aforementioned HPLC method. Encapsulation efficiency was calculated using following equation [28]:

\[
\text{Encapsulation efficiency} = \frac{\text{mass of drug added to the nanoparticles} - \text{mass of free drug}}{\text{mass of drug added to the nanoparticles}} \times 100\%
\]
In vitro drug release was determined at 37°C in phosphate buffered saline (PBS, pH 7.4) under sink conditions [28]. Samples were withdrawn at pre-determined time points and the amount of free drug released from the nanoparticles at each time point was determined by HPLC.

**Differential Scanning Calorimetry**

Thermal analysis by differential scanning calorimetry (DSC) was performed to confirm the presence of 2-ME in an amorphous state in the nanoparticle formulation. DSC analyses were performed using a Q200 DSC (TA Instruments, New Castle, DE). Samples containing 2–3 mg of drug powder or lyophilized nanoparticles were accurately weighed and hermetically sealed in aluminum pans. DSC scans were recorded at a heating rate of 10°C/min from 5°C to 215°C. During the thermal scans, the crucible was purged with nitrogen.

**In vitro cellular effects**

Immortalized human leiomyoma cells (HuLM) cells were a kind gift from Dr. Darlene Dixon [27]. Our group obtained them as a generous gift. The huLM cells were cultured as previously described [12, 28-29]. These cells maintain uterine fibroid cell characteristics without chromosomal abnormalities [30]. Briefly, the cells were grown in minimal essential medium (Gibco, Grand Island, NY) supplemented with fetal bovine serum (HyClone, Logan, UT), essential and non-essential amino acids (Gibco), minimum essential medium vitamin solution (HyClone), and penicillin-streptomycin (Mediatech, Inc., Manassas, VA). The cells were maintained at 37°C with 5% CO$_2$ and 95% relative humidity. The cells were seeded on 96-well plates at a cell density of 12,500 cells/cm$^2$, with 100 µL of cell culture medium per well, and cell viability assessments were initiated once the cells reached 70-80% confluence. At that point, test treatments were added to study cell viability after 48 hours and 72 hours. The treatments included 2-ME alone, 2-ME-loaded nanoparticles, and blank nanoparticles at 1 µM, 3.5 µM and 10 µM (concentrations of 2-ME alone or in nanoparticles; blank nanoparticles had polymer concentrations matching those of the drug-loaded nanoparticle treatments). Cell culture medium without phenol red and 0.1% (v/v) Triton X-100 were used as positive and negative controls, respectively. At the end of the incubation period, the WST-1 assay was performed following the protocol provided by the manufacturer (Roche Diagnostics, Mannheim, Germany) [30]. Briefly, treatments were removed and cells were washed with 100 µL of cell culture medium without phenol red and 110 µL of working solution was added to each well. After incubating at 37°C for 2 hours, absorbance was measured at 450 nm using a Vmax® microplate reader (Molecular Devices, San Jose, CA), with correction from the background signal at 650 nm.

**Tumor samples**

This study received approval by the University of Texas Medical Branch Institutional Review Board (IRB). Leiomyoma tumor samples were collected from patients undergoing hysterectomy for symptomatic uterine fibroids at the University of Texas Medical Branch after obtaining informed consent. Ultrasound was used to screen patients before surgery and histopathologic examination was done to confirm leiomyoma diagnosis after hysterectomy.
Animal model and treatment

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch. All procedures were performed in accordance with IACUC guidelines. The Patient-Derived Xenograft (PDX) leiomyoma mouse model was utilized as previously described [31, 33-34]. In brief, 6-week-old female immunodeficient NOG (NOD/Scid/IL-2Rγnull) mice were purchased from Taconic (Hudson, NY). Mice (n=10) were housed in standard cages and all procedures were done under sterile conditions. Patient-derived human fibroid tumors were xenografted bilaterally subcutaneously (Figure 1). One of the mice in the control group was found dead during the second week of the experiment. Isoflurane (1–2% by facemask) was used for anesthesia. Buprenorphine (0.05–0.1 mg/kg, subcutaneous, twice daily, then as needed) was given for post-operative pain control. Animals were randomized to receive 2-ME-loaded or blank (control, unloaded) PEGylated nanoparticles. The dose of 2-ME was 50 mg/kg, with the dose of the control nanoparticles matching the polymer concentration of the drug-loaded nanoparticles. The nanosuspensions were filtered with a 0.45 µm syringe filter prior to injection. The nanoparticles were injected intraperitoneally three times weekly, with tumor volumes measured after 28 days of treatment by caliper following hair removal. Tumors removed and weighed.

Immunohistochemistry

Fibroids were removed after animal sacrifice and fixed in 4% formalin solution. Paraffin sections were prepared from fixed tumors and immunohistochemistry was performed for trichrome, the cellular proliferation marker Ki67, the G1 phase checkpoint marker Cyclin D1, progesterone receptor (PR), and estrogen receptor (ER). Slides were evaluated by an experienced pathologist who quantified the expression of these markers using a standard scoring system as described previously [30].

Statistical analysis

Data were analyzed with two-tailed independent sample t-tests comparing the drug-loaded nanoparticles to the unloaded control (blank) nanoparticles. P < 0.05 was used as the cut-off for statistical significance.

Results

Nanoparticle characterization

Table 1 shows the characteristics of polymeric nanoformulations prepared with 5%, 10%, and 15% drug loading. Each of the nanoparticle formulations—including the blank nanoparticles—had similar Z-average particle size, polydispersity index (PDI), and zeta potential values. The nanoparticles prepared at 15% drug loading had the highest encapsulation efficiency and were selected for all subsequent experiments (i.e., drug release, DSC, cytotoxicity, and the in vivo studies). The drug release profile in Figure 2 shows limited gradual release over the first 72 hours. The minimal burst release observed confirms the high encapsulation efficiency. The DSC thermographs in Figure 3 show a melting peak for 2-methoxyestradiol at 188°C. However, this peak was not observed in the thermograph for the drug-
In vitro cellular effects

Figure 4 shows that the viability of the huLM cells was not significantly different when treated with free 2-ME in solution for either 48 hours (Fig. 4A) or 72 hours (Fig. 4B), as measured by the WST-1 assay. Similarly, the control blank (unloaded) nanoparticles did not show any effects on huLM cell viability. However, when the cells were treated with the 2-ME-loaded nanoparticles, the cell viability decreased in a dose-dependent manner. Nanoparticle treatments at 1, 3.5, and 10 µM resulted in significantly decreased cell viability as compared to the cell culture medium control, and nanoparticle treatments at 3.5 and 10 µM were also significantly different from the treatments with free 2-ME at these same concentrations, for both treatment times ($P < 0.05$).

Fibroid size and volume

Mice were treated with blank PEGylated nanoparticles (control) or PEGylated 2-ME-loaded nanoparticles thrice weekly for 28 days (Figure 1). Fibroid volume was significantly decreased by 51% in mice treated with the 2-ME nanoparticles at 28 days (two-tailed t-test, $P = 0.005$, Figure 5A). We also noted a trend toward decreased fibroid mass in the 2-ME treated mice at 28 days (Figure 5B) (two-tailed t-test, $P = 0.094$).

Histological evaluations

To determine whether treatment with the PEGylated 2-ME-loaded nanoparticles would lead to cellular changes in fibroids, we performed immunohistochemistry on fibroid samples from control-treated and 2-ME-treated mice after 28 days of treatment, as outlined in Figure 1. There was no significant difference in trichrome staining between the control and the 2-ME-treated mice, suggesting similar cellular composition of fibroids between groups (two-tailed t-test, $P = 0.659$). We observed a trend toward decreased Cyclin D1 expression in the 2-ME-treated group as compared to the control group, but it was not statistically significant ($P = 0.055$, Figures 6A, 6E). There was no significant difference in Ki-67 expression between groups (Figures 6B, 6E) nor in estrogen receptor expression (Figures 6D, 6E). However, progesterone receptor expression was significantly lower in fibroid samples from mice treated with the 2-ME-loaded nanoparticles compared to the control group ($P < 0.05$, Figures 6C, 6E).

Discussion

The optimal medical treatment modality for fibroids, a debilitating condition afflicting a large number of reproductive-age women, is still an active area of investigation. The principle of using PEGylated nanoparticles as a drug delivery system to uterine tissue has been successfully implemented for oxytocin receptor antagonists in a proof-of-concept in vitro study [32]. We recently described successful treatment of fibroids with liposome nanoparticles loaded with simvastatin in a xenograft mouse model [33]. Here,
we demonstrate for the first time the successful *in vivo* implementation of a potentially immediately translatable novel 2-ME nanoparticle treatment for uterine fibroids with demonstration of efficacy in an immunodeficient human leiomyoma xenograft mouse model. Although the Z-average particle diameter of these PEGylated 2-ME-loaded nanoparticles was smaller than the particle size of the non-PEGylated 2-ME-loaded nanoparticles which we had prepared previously, the high encapsulation efficiency confirmed by DSC and the drug release profile was similar [12]. In addition, the PEGylated 2-ME-loaded nanoparticles from this study caused significant reductions in the cell viability of huLM cells, just as had been observed for the non-PEGylated 2-ME-loaded nanoparticles investigated previously [12]. Our demonstration of a trend towards a decrease in Cyclin D expression suggests slower cell cycling in fibroid cells exposed to 2-ME. Likewise, the decreased progesterone receptor expression in fibroid cells exposed to 2-ME could suggest lower tumor receptivity to this hormone. The results point to a potential mechanism explaining the efficacy of PEGylated 2-ME for the treatment of fibroids and is consistent with previous *in vitro* work [34-36].

Nanoparticle-assisted therapy is an emerging innovative strategy to deliver drugs safely and at high tissue concentrations [37]. Several platforms within nanoparticle technology exist, each of which differ in terms of solubility, ease of synthesis, and *in vivo* potency/efficacy. These include liposomes, dendrimers, and polymeric nanoparticles [37]. Our group previously explored the liposomal formulation of 2-ME in uterine fibroids [31]. Here, we synthesized polymeric nanoparticles loaded with 2-ME in order to efficiently concentrate our compound of interest (2-ME) into a compact vehicle for delivery to fibroid tissue. Strengths of our model include the use of human leiomyoma tumor samples, significant tumor volume shrinkage without significant side effects or toxicities, and ease of dosing.

Limitations include the use of an immunodeficient mouse model, which may underestimate potential immunogenic or antigenic effects of our PEGylated nanoparticles, although we feel this is less likely given previous studies demonstrating a lack of a significant cytokine response to PEGylated compounds [38-39]. Moreover, the optimal route of administration remains to be determined, as intraperitoneal injections (used in the present work) may not be as tractable as a subdermal or intramuscular options. Future work should directly compare regular 2-ME administration with 2-ME-loaded nanoparticles *in vivo* for direct pharmacological comparison, both in terms of efficacy and safety/toxicity. A 2-ME nanoformulation may allow for absorption via the oral mucosa, which would likely be more tenable for patients who would need to receive multiple doses per week [1]. It could also be formulated into an intrauterine device (IUD). Still, the current model demonstrates proof-of-concept for a PEGylated nanoformulation encapsulating 2-ME as a potential novel therapeutic option for treatment of fibroids. The ideal candidate for such treatment will need to be addressed in future studies. For instance, whether PEGylated 2-ME is equally efficacious for fibroids located submucosally, intramurally, or subserosally remains unknown given the present work. We look forward to new developments with this developing technology regarding fibroid research and other areas of medicine.

**Table 1.** Physicochemical characteristics of the 2-methoxyestradiol-loaded nanoparticle formulations investigated in this study. The blank nanoparticles were prepared with the same PEGylated PLGA
polymer but without any 2-methoxyestradiol. The particle size is the Z-average diameter determined by dynamic light scattering. The data are presented as the mean ± s.d. (n=3).

<table>
<thead>
<tr>
<th>Drug Loading</th>
<th>Particle size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV)</th>
<th>Encapsulation Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (0%)</td>
<td>108 ± 0</td>
<td>0.14 ± 0.03</td>
<td>-30.1 ± 1.0</td>
<td>—</td>
</tr>
<tr>
<td>5%</td>
<td>104 ± 1</td>
<td>0.11 ± 0.02</td>
<td>-29.4 ± 0.6</td>
<td>97.9%</td>
</tr>
<tr>
<td>10%</td>
<td>112 ± 0</td>
<td>0.10 ± 0.09</td>
<td>-28.4 ± 0.3</td>
<td>98.9%</td>
</tr>
<tr>
<td>15%</td>
<td>106 ± 1</td>
<td>0.13 ± 0.02</td>
<td>-28.3 ± 1.1</td>
<td>99.1%</td>
</tr>
</tbody>
</table>

**Declarations**

Declarations of interest: none

**ETHICAL STATEMENT:**

- Ethics approval and consent to participate: Not applicable
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- Authors’ contributions: ER and MAB conceived of the work. SA-E, GWK, KV, JY, JS, MS, MM, SA, GK, ER, and MAB performed the experiments. SA-E and GWK wrote the initial draft of the manuscript. All authors edited the draft and approved the final version

- Acknowledgements: None

- Authors’ information (optional): None

**References**


**Figures**

**Figure 1**

Experimental design: (A) Schematic of the leiomyoma xenograft mouse model. (B) Administration paradigm for the 2-ME-loaded nanoparticles. (C) Representative histological fibroid image.
Figure 2

In vitro drug release profile of 2-methoxyestradiol from PEGylated PLGA nanoparticles at 37°C in phosphate buffered saline (pH 7.4). Results are expressed as the mean ± s.d. (n=3).
Figure 3

Differential scanning calorimetry thermographs of pure 2-methoxyestradiol, 2-methoxyestradiol-loaded nanoparticles, and blank (unloaded) nanoparticles. Samples were heated from 5°C to 215°C at a rate of 10°C per minute.
Figure 4

Cell viability of human uterine leiomyoma (huLM) cells treated with free 2-methoxyestradiol (2-ME) in solution, 2-methoxyestradiol-loaded nanoparticles (NPs), or blank (unloaded) nanoparticles at various concentrations of 2-methoxyestradiol, as measured by the WST-1 assay after treatment for 48 hours (A) or 72 hours (B). The blank nanoparticle treatments matched the polymer concentrations of the corresponding drug-loaded nanoparticle treatments. Triton-X 100 (0.1%, v/v) and cell culture medium
served as negative and positive controls, respectively, with data presented as percent of control (cell culture media treatments set as 100%). Results are expressed as the mean ± s.d. (n=6). *, \( P < 0.05 \) in comparison to the media control. #, \( P < 0.05 \) in comparison to the free drug at the same concentration.

**Figure 5**

Fibroid volume (A) and fibroid weight (B) after 28 days of treatment with 2-ME-loaded nanoparticles or control (unloaded nanoparticles). Fibroid volume was measured by caliper and fibroid weight was measured by postmortem scale in both groups. *, \( P < 0.05 \) by two-tailed unpaired t-test.
Figure 6

Histological evaluation of fibroids from mice treated with control (unloaded nanoparticles) or 2-ME-loaded nanoparticles. Representative immunohistochemistry of (A) cyclin D1 expression, (B) Ki-67 expression, (C) progesterone receptor (PR) expression, and (D) estrogen receptor (ER) expression. (E) Quantification of marker expression between the control-treated and the 2-ME-treated groups. *, $P < 0.05$ by two-tailed unpaired t-test.