Sustained Delivery of Tamoxifen for the Prevention of ER+ Breast Cancer Using a Nanofluidic Delivery Platform

Andrea Ballerini[1,2,‡], Carly S. Filgueira[1,‡], Eugenia Nicolov[1], Priya Jain[1], Giacomo Bruno[1], R. Lyle Hood[1], Francesco Scaglione[2], Alessandro Grattoni[1]*

[1]Department of Nanomedicine, Houston Methodist Research Institute, Houston, Texas, USA 77030;
[2]Department of Oncology and Onco-Hematology, University of Milan, Milan, Italy;
[‡]Denotes co-authorship

*Address correspondence to Dr. Alessandro Grattoni, Chairman and Assistant Professor, The Department of Nanomedicine, 6670 Bertner Avenue, Houston, Texas, 77030, USA. Email: Agrattoni@houstonmethodist.org

Abstract

Background: Tamoxifen (TMX) has been in clinical use for over 40 years and can be considered the standard for breast cancer chemoprevention in high-risk patients. Unfortunately, patient compliance to treatment is low due to systemic toxicity. **Objective:** The present study demonstrates the use of a nanochannel delivery system (nDS) for controlled and sustained local release of TMX in mammary tissue. **Method:** TMX release in vitro from the nDS was assessed using different concentrations of the solubilizer PEG400. The optimal configuration was then studied in 9-week old NMU-treated Sprague Dawley rats, and the pharmacokinetic profile of TMX analyzed with LC-MS/MS. Systemic and organ toxicity was evaluated. **Results:** Although highly water insoluble, we showed that through utilization of PEG400, we were able to sustain the release of TMX in vitro for 2 months. In vivo, we released TMX from nDS implants and reached relevant plasma concentrations (>50 ng/ml). Our study showed sustained low dose delivery of TMX over several months from the implant placed adjacent to the mammary gland, thereby minimizing whole-body exposure and associated side effects. Compared to the oral TMX treated group, the nDS-TMX group maintained higher body weight and showed lower uterine weight. Compared to the sham group, TMX treatment reduced the number of mammary gland aggregates without affecting liver weight. **Conclusion:** We demonstrate that the nDS is a valid technology for long-term delivery of TMX. The nDS is applicable for breast cancer prevention and may offer an avenue to reduce the incidence of estrogen sensitive breast cancer.

Keywords: Breast Cancer; Tamoxifen (TMX) ; Overectomy ; Nanochannel Delivery System (nDS); NMU; PEG400 ; Solubilizer; Drug Delivery

Introduction

A high incidence (~75%) of primary breast cancers are estrogen receptor positive (ER+), and a large fraction of these patients can pursue chemopreventive therapies. However, due to adverse side effects, only 5% to 20% of the tens of thousands of women at high risk who could benefit from chemotherapeutics enroll in preventive treatment. There is a clear need for alternative preventive strategies that minimize side effects and improve enrollment and compliance. Selective estrogen receptor modulators, such as tamoxifen (TMX), have been shown to reduce ER+ breast cancer incidence by up to 50% among high-risk women. TMX binds with high affinity to both ER subtypes[1] and has differential agonistic or antagonistic activity at target tissues throughout the body[2]. Importantly, along with raloxifene, TMX is one of only two FDA-approved drugs for breast cancer prevention. TMX has already been in use for over 40 years and has a proven record in pre- and post-menopausal women. However, the drug is marred by side effects, the most common being symptoms of menopause. Further, women treated systemically and chronically with TMX were found to have an increased incidence of endometrial carcinoma. Although considered rare, this side effect, along with other serious adverse effects (such as blood clots, strokes, and cataracts), has resulted in a debate concerning the benefits versus risks of TMX use in cancer prevention.

In 1998, the National Cancer Institute and the National Surgical Adjuvant Breast and Bowel Project co-sponsored a trial exploring TMX's potential as a breast cancer risk reducer. In high-risk women, the trial demonstrated a 49% reduction in the incidence of invasive ER+ breast cancer after treatment with TMX, resulting in its approval as a chemopreventative agent. However, significant barriers remain for TMX's widespread use as a result of its potential toxicities, such as endometrial cancer and thromboembolic events. In order to overcome these adverse effects to exploit the benefits of TMX, scientists are investigating alternative strategies ranging from low-dose regimens to topical administration[3,4] of its active metabolites to avoid excessive

systemic exposure and resulting toxicity. Therefore, an alternative method, which can introduce TMX locally at the cancer prevention site or at low concentrations to aid in the avoidance of system toxicity may dramatically increase the clinical utilization of TMX for breast cancer chemoprevention.[5]

As the key to success for breast cancer chemoprevention relies upon long-term delivery of drugs while circumventing side effects, we have developed a novel local delivery strategy for the constant and sustained administration of TMX. We maintain a long-term, local release of TMX in mammary tissues by utilizing a novel implantable nanochannel Delivery System (nDS). The nDS consists of a bioinert, implantable, and mechanically robust silicon membrane which houses an exact number of densely packed *slit*-nanochannels as small as 2.5 nm with tight tolerances on size, geometry, and surface properties.[6] Providing steady levels of TMX at the mammary gland target tissue through nDS delivery maximizes the therapeutic index while limiting the unwanted secondary effects, which will ultimately improve patient compliance.

In this work we chemically induced tumorigenesis in Sprague-Dawley rats by N-methyl-N-nitrosourea (NMU) injection to promote the development of estrogen-dependent tumors. NMU-induced tumors show strong similarities to the grade II tumors observed in majority of ER+ patients.[7] We then performed ovariectomies seven days after NMU injection to mimic post-menopausal biology. nDS implants loaded with either TMX or PEG400 (negative control) were inserted under the left abdominal mammary gland (number 4) to determine the effects of nDS-TMX on tumor growth and tumor biomarkers. Utilizing LC-MS/MS we were able to quantitatively determine the amount of TMX released from the nDS into the plasma. Rats were also examined for palpable tumors to assess breast tumor incidence, latency to onset, and multiplicity. Our results show that the nDS implant enables the effective delivery of TMX in this breast tumor model. Further, this technology has the potential to rapidly provide long-term breast cancer protection with significant improvement in the quality of life of patients at high risk, thereby saving thousands of lives every year.

Materials and Methods

In vitro release of TMX from the nanodelivery system (nDS)

The nanochannel membranes and drug delivery system used in this study are described elsewhere.[8,9] nDS membranes (NanoMedical Systems, Inc.) were assembled with implantable discoidal shaped titanium capsules[8] (Figure 1) loaded with TMX and filled with 50% PEG400 (1:1 PEG/PBS solution). PEG400 was adopted as a solubilizer to overcome the poor solubility of TMX. Tamoxifen citrate was purchased from Cayman Chemical. PEG400 was purchased from USB corporation. For the vitro studies 1.5 ml reservoir capsules were filled with 12 mg/ml TMX in either 75% or 50% PEG400. Implants were placed in low-actinic media bottles containing 40 ml of PBS sink solution and incubated at 37°C, under constant stirring (n=4). Samples (500 µl) were collected from each bottle daily, diluted in methanol (1:1 v/v), and stored at 4°C until analyzed. TMX content in the samples was measured by HPLC at 280 nm.

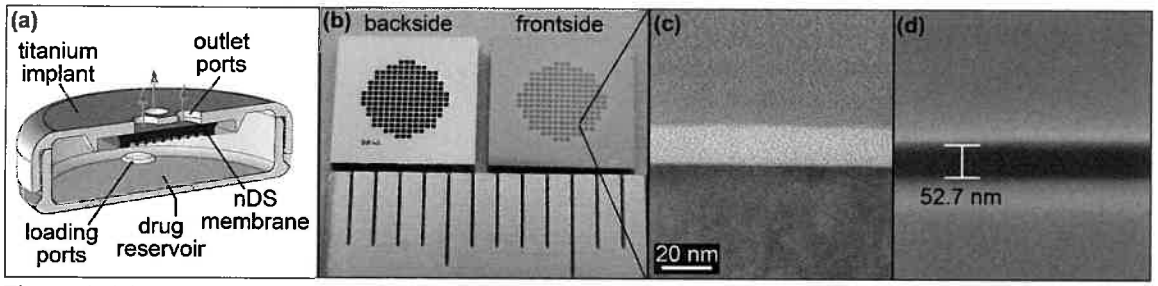

Figure 1. (a) 3D rendering of the nDS membrane assembled into the CP2 titanium implant, (b) top down photograph of the front and backside of the nDS membrane, (c) TEM image of a cross section of a 20 nm nanochannel, and (d) SEM image of of a cross section of a 50 nm nanochannel.

In vivo release of TMX from the nDS

For the in vivo work, implantable medical grade capsules designed to contain a 500 µl reservoir with 50 nm nanochannel membranes were fabricated in replicates and loaded with ~15.6 mg of TMX in a 1:1 PEG/PBS solution. This initial nanochannel membrane size was selected using a decision algorithm developed through extensive experimental analysis[8].

Animals and Treatments

Female Sprague-Dawley rats 40-44 days old (125-149 grams) were purchased from Harlan. The rats received two IP injections of N-nitroso-N-methylurea (NMU) (5 mg/kg body weight followed by 50 mg/kg body weight one week later at 54-58 days old) to stimulate the development of mammary tumor. It has been previously reported that the percent incidence of mammary carcinomas is higher and a greater number of carcinomas per rat with generally shorter latent periods occur when NMU is administered to rats at a younger age.[10] NMU was purchased from Spectrum Chemical Corp and dissolved in a solution of 0.9% saline that has had its pH lowered to 4.6 with acetic acid. The 0.9% sodium chloride was purchased from Sigma Aldrich. The NMU was kept on ice and mixed into the saline immediately prior to the injections. A homogenous solution was achieved by sonicating at room temperature for about 10 min.

NMU injection was performed 7 days before ovariectomy and nDS implantation. Implants containing nDS devices loaded with TMX or vehicle (1:1 PEG400/PBS solution) were subcutaneously inserted underneath the left abdominal mammary gland #4 (Figure 6a modified from P. Sun[11]) using aseptic technique. Rats were divided into 5 groups (15 rats/group) and received over 5 months of treatment as follows: group 1 sham ovariectomy, (sham); group 2, ovariectomized (control, not treated) d; group 3, ovariectomized and implanted with nDS-PEG400 (nDS-PEG400); group 4, ovariectomized and implanted with nDS-TMX and 100% PEG400 (nDS-TMX); group 5, ovariectomized and treated with TMX by delivery in food (oral TMX). The oral TMX delivery group was fed Teklad 2020X Rodent Chow with 23.6 mg/kg TMX purchased from Research Diets, Inc., for a target dose of about 500 µg/day. We estimate the actual dose of oral TMX over the course of the study to range from 270 to 330 µg/day by weighing the total amount of food distributed to the animals weekly.

The experimental setting used for this study is described as follows. Nineteen days before implantation the animals were weighed and blood collected by saphenous vein for baseline levels. Blood drawn was analyzed for plasma TMX level to assess baseline. On day 0, the rats were ovariectomized (groups 2-5) or sham ovariectomized (group 1, leaving ovaries intact) and implanted with one nDS device each. Implants containing nDS membrane loaded with TMX were inserted onto the left abdominal gland and sutured in place on both sides of the device (Figure 6a.b). Blood was collected from the rats at specified intervals (Day 0, 1, 3, 7, weekly during the first month, every other week for the second month, and then monthly for 6 months), and the rats were examined weekly for palpable tumors, body weight, and morbidity. Tissues, including mammary fat pad, uterus, liver, and tumors were collected. The implant was removed and the area surrounding the implant checked for scar tissue. SuperChem diagnostic profiles were performed by Antech Diagnostics to obtain a comprehensive blood chemistry panel of the animals at the time of euthanasia. All animal experiments were approved by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute.

LC-MS/MS

Drug concentrations were measured in blood by means of HPLC tandem Mass Spectroscopy. Plasma samples were prepared for LC-MS/MS by first thawing on ice. Next, 75 µl of ice cold acetonitrile with 25 ng/ml 7-hydroxycoumarin was added to 25 µl of thawed plasma. The samples were vortexed and placed on ice. 50 µl of 0.2% formic acid in water containing 25 ng/ml 7-hydroxycoumarin was added. Samples were analyzed using a Waters Xevo TQ-S triple quadrupole mass spectrometer with Waters Acquity UPLC and temperature controlled sample manager.

Statistical analysis

GraphPad Prism 5 software was used for all statistical analyses. Differences between groups were analyzed using one-way ANOVA with Bonferroni post test (α=95%) or Student's two-tailed test. Data are expressed as the mean ± SEM. Symbols denoting P values and sample sizes are indicated in each figure legend.

Results and discussion

To better understand which nanochannel size and solubilizer concentration would aid in TMX release for our consequent in vivo study, we performed in vitro release testing using three different settings (Figure 2). We have previously established a design algorithm for therapeutic agents to aid in the selection of the best nanochannel configuration to release various compounds based on their dimensions, solubility and net charge at neutral pH.[8] Due to its hydrophobicity (Log D > 3), the release of TMX in an aqueous environment requires a hydrophilic solubilizer. We solubilized lipophilic TMX with different concentrations of PEG400, a highly hydrophilic polymer widely used in a variety of pharmaceutical formulations. In previous studies, we used a similar approach and delivered pharmaceuticals from the nDS to an aqueous environment through the use of inclusion complexes such as docetaxel with macrocyclic oligosaccharide[12,13] and have performed in vitro release testing with resveratrol and atorvastatin[9]. A burst release during the first week of testing was observed. This was attributed to the high concentration gradient of PEG400 across the nanochannel membrane and a related transient increase

in osmotic pressure in the drug reservoir. Expectedly, the burst release was reduced by diminishing the amount of PEG400 in the drug reservoir or by increasing the nanochannel size from 20 to 50 nm. After a few days the release of TMX stabilized and remained constant at a rate of ~30 µg/day for 8 weeks (Figure 2). We, therefore, chose the 50 nm membrane configuration and 1:1 PEG400/PBS reservoir solution for the in vivo work as it did not demonstrate a large burst release profile and adequately sustained TMX levels for a duration of time.

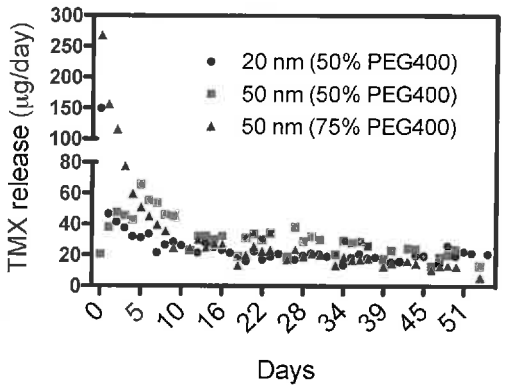

Figure 2. Releas[illegible] th 20 nm membranes loaded with 50% PEG400 (back circles), 50 nm membranes loaded with 50% PEG 400 (red squares), and 50 nm membranes loaded with 75% PEG400 (blue triangles).

To prove the effectiveness of the nDS in sustaining the delivery of TMX, we analyzed the plasma concentration of the compounds over the course of the study. Figure 3 shows the sustained delivery over 40 days. No TMX was seen in the nDS-PEG400 animals as expected, while the TMX concentration was higher at each measured time point in the oral group compared to the amount released by the device (nDS-TMX). This result is in line with our expectation of a sustained low release of TMX, exposing the treated animal to lower systemic concentration and reducing the risk of side effects due to the chronic exposure. Kisanga *et al.*[14] measured serum and tissue concentrations of TMX and three of its metabolites and showed a dose-concentration relationship. The values that they obtained after administering TMX at oral doses of 1, 5, and 20 mg daily are in line with the results we obtained in the nDS-TMX group. This proves the capability of the nDS to achieve clinically relevant concentration for an extended period of time, which can be prolonged according to the amount of TMX loaded and the size of the reservoir. Moi *et al.*[15] performed rodent experiments using a higher oral dose (40 mg/kg) for 14 days and showed a median TMX concentration in plasma of 203 ng/ml, while in the tumor they saw fifty-time higher values. The intratumoral accumulation of the selective estrogen receptor modulator (SERM) is due to the presence of estrogen receptor alpha.

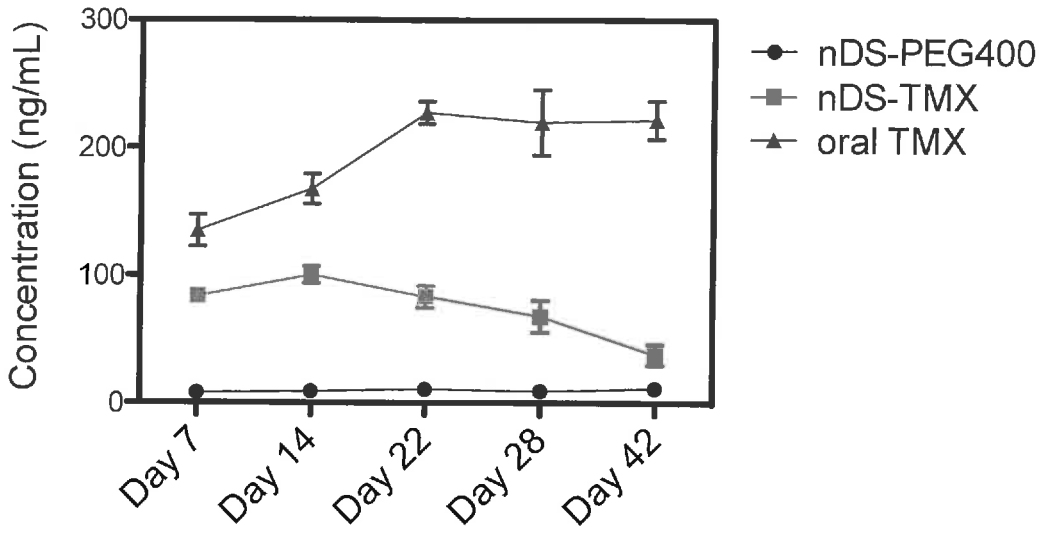

Figure 3. Total plasma tamoxifen measured LC-MS/MS for rats treated with nDS-PEG400 (back circles), nDS-TMX (red squares), and oral TMX (blue triangles).

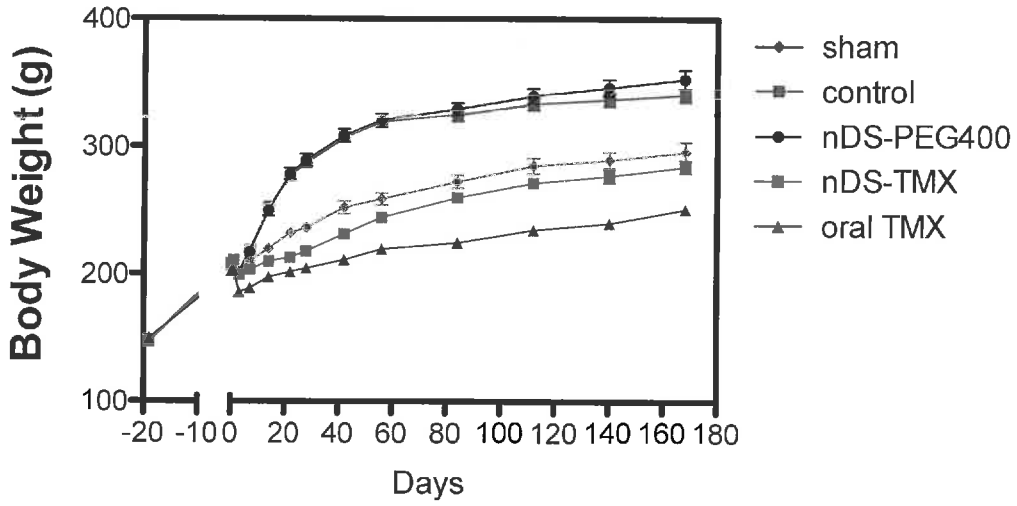

Figure 4. Body weight (n = 15) for sham, control, nDS-PEG400, and nDS-TMX, and oral TMX treated rats.

Figure 4 shows the body weight over the course of the study (where the weight of the device has been taken into consideration and subtracted from the weight of the rats in the nDS PEG400 and nDS TMX groups). The control (ovariectomized) group gained weight at a faster rate when compared to the sham group likely due to lower metabolic and activity rate.[16] We did not see any differences in weight between the control (not treated, ovariectomized) group and nDS-PEG400 group, indicating that the implantation of the device does not significantly affect weight. In the groups treated with TMX we saw greater weight loss in the oral group probably due to 1) higher systemic exposure to the drug and 2) an observed reduction in food intake. Similar reductions in body weight, growth rate, and food consumption due to TMX and other ERα agonist treatment has been observed by others.[15,17–19]

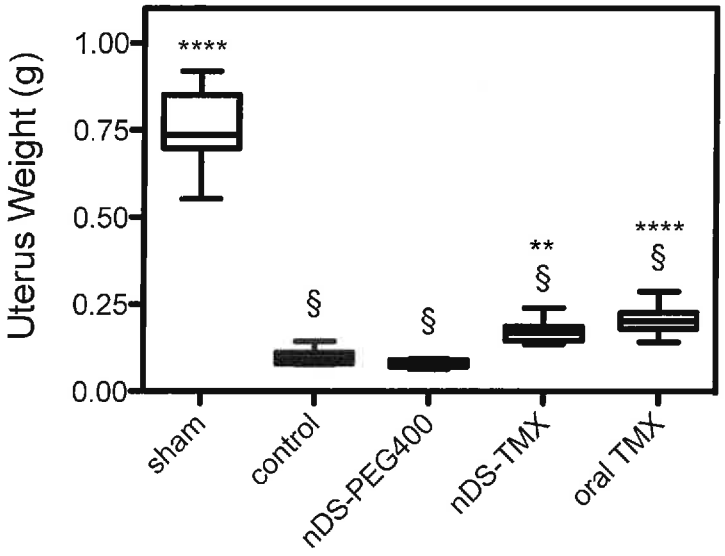

Figure 5. Uterus weight (n = 15) for sham, control, nDS-PEG400, and nDS-TMX, and oral TMX treated rats.

Figure 5 shows that the presence of ovaries (sham group) highly influences the weight of the uterus as expected. We observed atrophic uteri in the ovariectomized groups (control, nDS-PEG400, nDS-TMX, and oral TMX) leading to decreased weight values when compared to the sham. The TMX treated groups (nDS and oral) have different levels of hyperproliferative action due to the pharmacological effect of the SERM on the uterus.[20,21] This is a side effect of TMX. We see significantly higher effects in the oral group due to different dose and systemic exposure.

While TMX is strongly antiestrogenic in the mammary epithelium, hence its use in both the prevention and treatment of breast cancer, in uterine epithelium, TMX is proestrogenic. Women treated systemically and chronically with TMX were found to have an increased incidence of endometrial carcinoma.[22] Although considered rare, this side effect, along with other serious adverse effects (such as blood clots, strokes, and cataracts), has resulted in a debate concerning the benefits versus risks of TMX use in cancer prevention.[23] Minimizing these adverse side effects through use of an alternative preventive strategy, such as the nDS, may increase the percentage of women at high risk for breast cancer who enroll in preventive treatment in addition to improving treatment compliance.

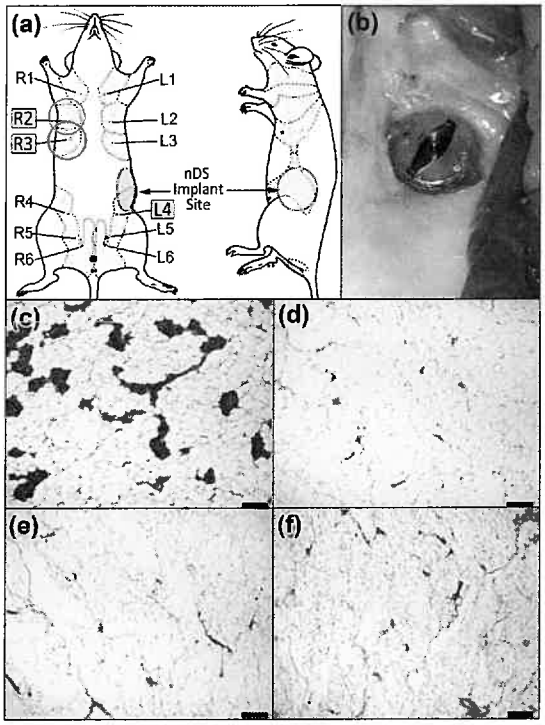

Figure 6. (a) Diagram of a study animal and site of nDS implantation showing distal mammary glands (left #2/3). (b) Photo of device implantation in rats showing the fibrotic capsule surrounding the nDS implant. Representative H&E staining of the mammary fat pad obtained from inguinal glands opposite to the device implantation site for (c) sham, (d) nDS-PEG400, (e) nDS-TMX, and (f) oral TMX. Magnification 4x. Scale bar is 200 µm.

It is not surprising that visible and palpable tumors were first evident in the sham group (Day 63 after device implantation). NMU induced tumors in the sham group ranged from 5-15 mm in size as measured using a caliper. It is well established that 85-90% of chemically induced mammary tumors in rats will disappear or diminish significantly in size after the ovaries are removed from the animal.[24] H&E staining was performed on the mammary fat pad obtained from inguinal glands opposite to the device implantation site (Fig. 6c-f). While all animals were treated with NMU, the Sham group showed a higher prevelance of hyperproliferative clusters in the mammary fat tissue (Figure 6c) even in animals and areas where no palpable tumors were present. The other groups (nDS-PBS, nDS-TMX, and oral TMX) did not show histological evidence of lobular units, although the oral-TMX group did show more ductal branching possibly due to the reduced distribution of adipose tissue (Fig. 6d-f).

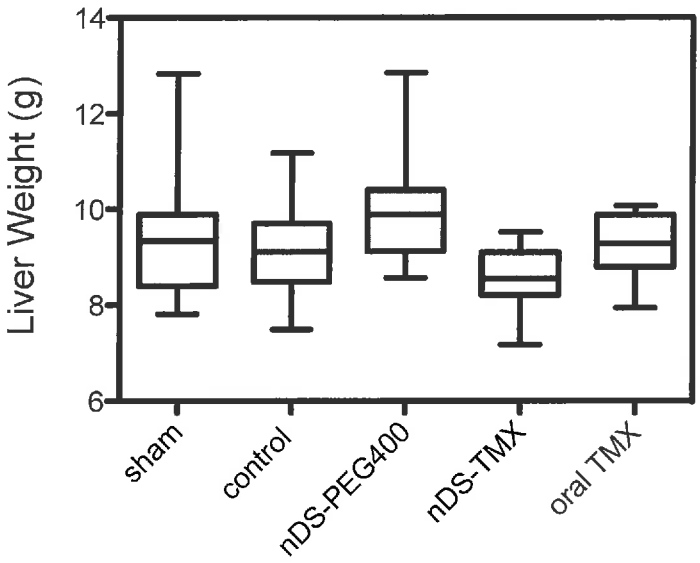

Figure SI1. Liver weight (n = 15) for sham, control, nDS-PEG400, and nDS-TMX, and oral TMX treated rats.

Liver cancer is a major rat-specific side effect of TMX. It is usually evidenced with large doses (3.3 mg/kg) and prolonged administration (>24 weeks).[25] We show that liver weights did not change across the sample groups (Supporting Information Fig. 1). Moreover, macroscopic anatomical inspections of the liver did not show any evidence of tumors. This data validates that the dose we have chosen remained low enough to not cause complications such as the development of liver hypertrophy or hepatoadenoma in rats. The literature supports this results as Kafkasli *et al.*[19] did not notice any histopathological changes in the liver tissue of rats treated for over 2 months with TMX dosed at 0.8 mg/kg. We also sampled the blood at sacrifice from each group for biochemical analysis and did not notice any differences in liver and kidney markers, such as bilirubin, GGT, albumin, cholesterol, triglyceride, creatinine, and BUN.

Conclusions

TMX has been studied for years as both an adjuvant therapy for breast cancer patients and a chemopreventative for healthy women with a high risk for breast cancer.[26] A previous meta-analysis of 55 clinical trials involving 37,000 early-stage breast cancer patients showed reductions of 47% in recurrence and 26% in mortality with adjuvant TMX therapy for 5 years.[27] These benefits led to FDA approval of TMX for adjuvant use in 1990 and to subsequent studies for its role in chemoprevention. In the National Surgical Adjuvant Breast and Bowel Project Breast Cancer Prevention Trial (NSABP-P1), which enrolled 13,388 women aged 35 years or older who were at an increased risk for breast cancer, 5 year TMX treatment reduced the risk of both invasive and noninvasive breast cancer by approximately 50% and overall mortality by 19%.[28] This effect was more evident in women with hormone receptor–positive disease and confirmed in the International Breast Intervention Study (IBIS-1).[29] These studies then led to TMX approval in 1998 for use in preventing breast cancer in premenopausal and postmenopausal women who are at an increased risk for the disease. Nevertheless, long-term TMX treatment is limited by its associated adverse effects. Indeed, 23.7% of the TMX arm versus 19.7% of the placebo arm discontinued treatment by year 4.5 in NSABP-P1 trial, and 5 year compliance was 64% in the TMX arm versus 74% in the placebo arm in IBIS-1 ($p < 0.001$). Land *et al.*[30] showed that the compliance to TMX treatment reduces with time from 91% at 1 month to 79% at 36 months, with only 41% at full adherence. Taken together, these trials demonstrate the need for alternative solutions to increase treatment compliance and maximize the chemoprevention or adjuvant hormone therapy benefits.

Our study demonstrates the feasibility to develop the nDS for releasing TMX in different experimental settings. While in vitro testing is useful to select the best nanochannel conformation and solubilizer to achieve constant release and avoid an initial burst profile associated with acute toxicity, in vivo testing is critical. Our devices were

well tolerated in rats and minimal fibrotic tissue was observed surrounding the implant, confirming previous results of prolonged nDS implantation[31]. Pharmacokinetic analysis confirmed the validity of the nDS in delivering sufficient TMX to reach clinical relevant plasma concentrations.
Rats treated with TMX did not have an increase in body weight at the same rate or scale as the other groups. This may be attributed to TMX's effect on both food intake and weight gain. Uterus weight was lower in the ovariectomized rats, but significantly higher in those groups treated with TMX, which may be a result of hyperplasia. Although long-term TMX treatment in rodents has been found to be associated with liver cancer,[17,32] liver weights were not affected by TMX treatment. TMX was effectively released through our nanochannel device; however, more studies are needed to improve drug solubility to achieve a longer and more constant release needed for treating larger animals and humans. The nDS has the potential for significantly improving breast cancer prevention and the quality of life of patients at high risk, thereby saving thousands of lives every year and increasing compliance to chronic hormone treatment.

Declarations

Authors' contributions

AB and CSF carried out the experimental work and drafted the manuscript. EN, PJ, GB, and RLH carried out the experimental work. FS participated in coordination and helped to draft the manuscript and interpret the data. AG conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests

AG discloses a financial interest in NanoMedical Systems, Inc. All other authors declare no conflict of interest.

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