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Preclinical and Clinical Evaluation of Intraductally Administered Agents in Early Breast Cancer

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Abstract

Most breast cancers originate in the epithelial cells lining the breast ducts. Intraductal administration of cancer therapeutics would lead to high drug exposure to ductal cells and eliminate preinvasive neoplasms while limiting systemic exposure. We performed preclinical studies in *N*-methyl-*N*′-nitrosourea–treated rats to compare the effects of 5-fluorouracil, carboplatin, nanoparticle albumin-bound paclitaxel, and methotrexate to the previously reported efficacy of pegylated liposomal doxorubicin (PLD) on treatment of early and established mammary tumors. Protection from tumor growth was observed with all five agents, with extensive

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Author contributions: V.S. conceived the clinical study, supervised the regulatory submission, analyzed the data, supervised the clinical procedures, and wrote the manuscript. T.M. designed, conducted, and analyzed the preclinical experiments and performed the in vivo binding assays. T.Y. assessed mammary gland whole mounts. S.L.K. performed preclinical experiments and assisted in analysis. Z.Z. designed, performed statistical analysis for preclinical and clinical studies, and wrote descriptions of the statistical methods. E.G. performed histopathological evaluation of clinical study specimens. D.L.H. performed histopathological evaluation of preclinical study specimens. M.A.R. performed pharmacokinetic analysis. S.S. designed, supervised, analyzed data, and wrote descriptions of the preclinical project. V.S., L.K.J., N.F.K., S.J., P.P., K.T., R.J.B., J.R.L., and T.N.T. enrolled and monitored study patients and performed study procedures. All authors reviewed and approved the final manuscript.

Competing interests: S.S. has served as a consultant to Windy Hill Medical (Los Angeles, CA).

Accession numbers: The clinical trial has been registered and description of the design can be found on http://www.clinicaltrials.gov (NCT00290732).

SUPPLEMENTARY MATERIAL
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epithelial destruction present only in PLD-treated rats. Concurrently, we initiated a clinical trial to establish the feasibility, safety, and maximum tolerated dose of intraductal PLD. In each eligible woman awaiting mastectomy, we visualized one ductal system and administered dextrose or PLD using a dose-escalation schema (2 to 10 mg). Intraductal administration was successful in 15 of 17 women with no serious adverse events. Our preclinical studies suggest that several agents are candidates for intraductal therapy. Our clinical trial supports the feasibility of intraductal administration of agents in the outpatient setting. If successful, administration of agents directly into the ductal system may allow for "breast-sparing mastectomy" in select women.

INTRODUCTION

Treatments for local control of breast cancer range from limited surgical excision to a mastectomy, with or without radiation therapy. These treatments alter breast appearance, affecting body image and quality of life. To reduce risk of distant recurrence and death, most women with invasive breast cancer are also offered adjuvant systemic therapy, which often includes polychemotherapy for several months or, for those with a hormone receptor–positive disease, endocrine therapy for 5 to 10 years. The adjuvant systemic therapy administered to those with invasive disease is critical to reduce risk of systemic recurrence. Ductal carcinoma in situ (DCIS) incidence in the United States rose from 1.87 per 100,000 in 1973 to 1975 to 32.5 in 2004 (1). Women with DCIS are offered local therapy and, often, 5 years of endocrine therapy. The few options that are currently available for breast cancer prevention or for treatment of premalignant disease are limited to prophylactic mastectomy, surveillance, or 5 years of endocrine therapy. Treatments with these endocrine agents are associated with major side effects that can be life-threatening. Interventions that are minimally invasive without systemic toxicity would be desirable for women with intraductal or premalignant lesions or for those at high risk.

The "sick lobe" theory (2) suggests that breast carcinoma is a lobar disease, not just confined to the visible ductal or lobular structures. If this theory is valid, surgical removal by lumpectomy is not sufficient. Intraductal administration of active agents could be an attractive option for DCIS or premalignant lesions, such as atypical ductal hyperplasia or lobular carcinoma in situ, because it can target the entire tree of the cancer-containing lobe.

Most breast cancers originate in the epithelial cells lining the duct. We hypothesized that administration of agents directly into the breast ductal system through the nipple (intraductal) could prevent the development of new tumors or the progression of preinvasive neoplasms. Indeed, we have previously demonstrated that the administration of intraductal pegylated liposomal doxorubicin (PLD) in two preclinical models—the *N*-methyl-*N*′-nitrosourea (MNU)–induced rat mammary tumor and the spontaneously arising Her2/*neu* transgenic mouse mammary tumor—is associated with a reduction in tumor volume, eradication of premalignant disease, and prevention of new lesions (3). Although the concept of intraductal therapy has been attractive for decades, several challenges have been identified and must be overcome before it can be ready for clinical use (4), including the need for effective technological tools, selection of appropriate biomarkers and treatment targets, patient acceptance of ductal access, proper patient selection, and appropriate ductal targets.

Here, we have expanded our previous investigations to determine the effects of intraductal administration of other anticancer agents, including 5-fluorouracil (5-FU), carboplatin, nanoparticle albumin-bound paclitaxel (nab-paclitaxel), and methotrexate, on treatment of early and established tumors in a chemically induced rat carcinogenesis model. The observed effects were compared to those mediated by PLD. While expanding the preclinical

studies, we also initiated a phase 1 clinical trial to establish the logistics of intraductal administration in an outpatient setting to women with breast cancer awaiting a mastectomy. We chose PLD for the clinical trial because it was the first agent that demonstrated marked reduction of tumor formation in our laboratories.

RESULTS

Therapy of early lesions in rodents

Female Sprague-Dawley rats administered the direct-acting carcinogen MNU develop preinvasive neoplasms in the mammary glands within 21 days and multiple palpable mammary tumors with a latency of 4 to 6 months (5, 6). The MNU-treated female rats develop mammary carcinomas that are 70% estrogen receptor (ER)–positive and 30% ER-negative (the same animal can bear both ER-positive and ER-negative tumors). Nearly 90% of the tumors contain the *H-ras* oncogene (5), irrespective of ER status. Tumors can develop in all 12 mammary glands over time and may metastasize to the lung. We selected cytotoxic agents (carboplatin, nab-paclitaxel/Abraxane, and PLD) and DNA-damaging antimetabolites (5-FU and methotrexate) to test the intraductal route, because these are commonly and successfully used for systemic treatment or to enhance local therapy of breast cancer.

High doses of commonly used cytotoxic agents can be better tolerated when administered intraductally than intravenously, owing to lower systemic exposure (reduced toxicity) (3). To test this concept, we conducted a short-term pilot experiment consisting of groups of five rats (60 mammary glands total) that received either no treatment, saline intraductally, the human equivalent dose of 5-FU (12 mg, 1×), or higher than the human equivalent doses of carboplatin (4 mg, 1.5×), methotrexate (4 mg, 2×), or nab-paclitaxel (5 mg, 1.5×). These drugs were administered once intraductally to all 12 mammary glands. The cytotoxic agents were administered 2 weeks after MNU induction, and the rats were followed for 7 weeks after treatment. The administration of 1.5- to 2-fold higher human equivalent single intraductal doses of carboplatin, methotrexate, or nab-paclitaxel did not result in significant changes in body weight (fig. S1) compared to saline and no-treatment controls. The number of benign and preneoplastic lesions in the mammary glands, including palpable invasive ductal carcinoma (IDC), mammary intraepithelial neoplasia (MIN), and hyperplasia, counted in the whole mount and in the stained sections, was lower in each of the treated groups compared to untreated controls (table S1). The reduction in the number of IDC and hyperplasia lesions in the mammary gland was most significant in 5-FU–treated rats (5 lesions; $P < 0.05$) compared to saline-treated rats (17 lesions) and untreated animals (16 lesions) among 20 glands examined by dissection per group. No differences were observed in the number of visible and microscopic lesions between saline-injected and untreated rats (table S1). A separate experiment consisting of a long-term 15-week follow-up of additional MNU-administered rats (five rats injected intraductally with saline and five untreated rats) confirmed this finding: We did not observe a difference in tumor incidence between rats treated with intraductal vehicle (saline; 30 of 60 mammary glands) and untreated rats (27 of 60 mammary glands) (table S1). Untreated rats were therefore used as the negative controls in subsequent experiments.

Gross and histopathological alterations in treated rat mammary glands

We examined both gross changes and histopathology of the intraductally treated mammary glands in rats in the pilot experiment described above. At 7 weeks after intraductal treatment, we did not observe typical external manifestations of drug toxicity and adverse effects, such as hair ruffling, ulceration, or change in behavior, in any of the treated animals. 5-FU–treated rats had thinning of the coat owing to hair loss, probably from systemic drug

exposure. PLD-treated rats developed mild transient hair loss and skin erosions around the nipple, probably due to irritation from drug leakage in the small mammary glands (table S1).

Whole-mount analyses were performed with hematoxylin–stained third and fourth mammary glands (Fig. 1). Compared to untreated mammary glands, we observed a loss of density of ductal outgrowth in mammary glands treated with PLD (Fig. 1A, ×10 magnification). At higher magnification (×100), we observed no difference in ductal morphology in rats treated with 5-FU, carboplatin, methotrexate, or nab-paclitaxel compared to untreated rats (none) (Fig. 1A). Only the PLD-treated mammary glands displayed distended mammary ducts compared to untreated controls (Fig. 1A).

Histopathological examination of rat mammary glands revealed no alteration in the ductal structure for rats treated with 5-FU, carboplatin, methotrexate, or nab-paclitaxel compared with untreated controls (none) (Fig. 1A). However, we observed changes in the PLD-treated ducts, including a loss of ductal polarity, intercellular edema, and mild periductal fibrosis compared to untreated mammary glands. More detailed analysis by counting the number of ducts on hematoxylin and eosin (H&E)–stained sections of the fourth mammary glands from four rats in each group (16 fields, four sections per group) revealed that ductal number occurring singly or in terminal ductal lobular units was significantly lower in rats treated with carboplatin and PLD (table S2) compared to the untreated and saline-treated rats.

To assess epithelial proliferation in the mammary glands after treatment, we performed immunohistochemical analysis using the proliferation marker Ki-67. Seven weeks after intraductal treatment, the Ki-67 labeling index was significantly lower in carboplatin- and 5-FU–treated mammary glands compared to untreated controls (Fig. 1B and table S3). The level of Ki-67 after nab-paclitaxel and methotrexate was not statistically different compared to controls. A significant reduction in Ki-67 labeling index was not observed in PLD-treated mammary glands compared to untreated rats (Fig. 1B and table S3), despite the drastic reduction in number of ducts and greater distention observed in the PLD-treated duct (Fig. 1A).

On the basis of the safety profile generated above, we next tested administration of single intraductal doses of each agent to all 12 mammary glands and monitored the animals for 22 weeks after drug administration. Rats received carboplatin (6 mg), methotrexate (4 or 10 mg), nab-paclitaxel (5 mg), or no treatment. We observed a significant reduction in tumor frequency (Table 1) as well as delayed time to tumor development in the carboplatin-treated group compared to no treatment and to methotrexate- and nab-paclitaxel–treated rats ($P < 0.001$, Cox proportional hazards model with Bonferroni adjustment for multiple testing) (Fig. 1C).

Our pilot experiment has also suggested superior efficacy of 5-FU in reducing benign and malignant tumors in MNU-treated rats compared to other agents (table S1). We therefore compared the effects of intraductal 5-FU ($n = 11$) with intravenous 5-FU ($n = 10$) administered 2 weeks after MNU induction. In the rats treated with intraductal 5-FU, four mammary glands per animal (third to the fifth on the right side) were injected with 5-FU four times at weekly intervals, whereas eight mammary glands remained untreated. Rats receiving intravenous 5-FU followed the same dosing regimen. Twenty-two weeks later, we observed a significantly greater reduction in tumor incidence [7 of 44 (16%)] in the intraductal 5-FU–injected mammary glands and also in the uninjected glands [25 of 88 (28%)] of the same rats compared to rats treated with an equivalent dose of intravenous 5-FU [59 of 120 (49%)] on the same regimen (Table 2 and Fig. 1D). Intraductal 5-FU was significantly protective ($P = 0.018$, Cox hazard ratio model, Bonferroni adjustment for

multiple testing) when compared to untreated rats [103 of 240 mammary glands (57%)] (Fig. 1D).

Treatment of established mammary tumors in rats

In rats with early lesions, the total number of hyperplasias, MIN, or invasive carcinomas was the lowest after intraductal 5-FU administration compared to methotrexate, carboplatin, and nab-paclitaxel (table S1). Therefore, we tested the therapeutic effects of 5-FU in rats bearing established MNU-induced mammary tumors ranging from 125 to 200 mm^3. Rats ($n = 28$) were randomized to treatment with 1 mg of 5-FU administered intraductally to the tumor-bearing gland or the same dose intravenously once per week for 4 weeks. The mean tumor burden for each treatment group was measured over the course of 6 weeks (Fig. 1E). Six weeks after drug administration, all 14 animals in the intravenous group were euthanized owing to excessive tumor growth. In contrast, tumors in 10 of the 14 rats in the intraductal 5-FU group regressed completely. Of the tumors that did not regress in the intraductal 5-FU–treated group, two tumors remained stable and two grew slowly to a size of 10 mm^3 by the end of the same 6-week observation period. The average tumor volume in intraductally treated rats was significantly lower compared to those in intravenously treated rats over time, with the volume difference reaching a maximal point at 6 weeks (estimated mean difference = 1615.6 mm^3) (Fig. 1E and table S4).

Phase 1 clinical trial of intraductal PLD in women awaiting mastectomy

From February 2006 to October 2009, 19 women signed a written informed consent, and 17 women were enrolled in the intraductal portion of the study, yet 2 were ineligible owing to a low absolute neutrophil count. Three additional women receiving standard intravenous PLD were enrolled in a pharmacokinetic control portion of the study and signed a separate informed consent. The total number of women involved was 20, most of which were Caucasian and had been diagnosed with invasive carcinoma (table S5). Duct cannulation was successful in the 15 participants eligible for the intraductal portion of the study. In one woman, a duct was successfully cannulated; however, PLD could not be instilled, likely because of extensive disease. In another woman, cannulation was not successful probably owing to a previous excisional biopsy.

All planned dose levels, including dextrose alone (dose level 0) and 2, 5, or 10 mg of PLD (dose levels 1 to 3, respectively) per duct, were completed without serious adverse events, such as blood toxicity, skin toxicity, or pain, using the National Cancer Institute Common Terminology Criteria Adverse Events (CTCAE) version 3.0 (Table 3). Adverse events were minimal, including transient mild breast fullness (60%) and breast or nipple pain or discomfort (93%) (Table 3). Using a visual analog scale, we obtained a median breast/nipple pain score at baseline of 0.02 (range, 0 to 0.3) on a scale of 0 to 100 mm (with 0 mm representing “no pain” and 100 mm representing “most severe pain”). During the study, pain scores were 0.64 (range, 0 to 2.2) during nipple aspiration, 1.37 (range, 0 to 6.2) during the mammogram portion of the ductogram, and 0.91 (range, 0 to 2.3) during PLD administration. Examples of ductogram images from two separate women who completed preoperative standard chemotherapy with excellent responses as noted by little or no residual disease at the time of their surgery are shown (Fig. 2, A and B).

PLD pharmacokinetics

Our hypothesis was that plasma concentrations of doxorubicin after intraductal administration would be lower compared to the expected concentrations after intravenous administration of the same or higher doses. In theory, the doxorubicin and doxorubicinol that are found in the plasma are free from the liposomal formulation because only nonliposomal (unencapsulated) drug will escape the reticuloendothelial system. In addition,

only free (non–protein-bound) doxorubicin would be metabolized to doxorubicinol. Therefore, we measured plasma drug concentrations serially until the time of mastectomy to assess the systemic exposure of doxorubicin and doxorubicinol after intraductal administration. Plasma concentrations as early as 4 hours and up to 2 weeks after drug administration were lower in women who received intraductal PLD compared to those who received the agent intravenously (Table 4). Conversely, drug concentrations in the breast were considerably higher in women who received intraductal PLD compared to those administered intravenous PLD (Table 4).

We measured drug concentrations in the area of the mastectomy specimen that was dyed blue (presumably exposed to PLD) and in a nonblue area (associated with lower or no concentration of PLD). Doxorubicin concentrations were not always highest in the blue-dyed region, possibly owing to variability in the length of time between PLD administration and the day of surgery, or cannulation of an incorrect duct at the time of surgery. Contralateral breast tissue was available from seven participants, and nipple aspirate fluid was obtained from the breast that was selected for intraductal administration in three patients. We did not detect doxorubicin in either contralateral breast tissue or nipple aspirate fluid. Local exposure was not noted consistently until the 10-mg dose level in both the treated and the untreated regions. We detected low concentrations of the drug (doxorubicin) but not the metabolite (doxorubicinol) in breast tissue obtained from one of the three women who received PLD (40 mg/m^2) intravenously, despite high plasma concentrations (Table 4). In summary, administering low doses of PLD intraductally results in more drug in the breast than in the circulation—the opposite of what was observed for intravenous administration.

Substantial pathological changes, including inflammation, were not observed on routine microscopic examination of mastectomy specimens obtained from women who received intraductal PLD. At the highest dose level (10 mg of PLD), we observed reactive epithelial changes in the duct usually resulting from cytotoxic chemotherapy, including focal epithelial necrosis in the treated (Fig. 2C) but not in the untreated (Fig. 2D) regions. These changes were consistent with those seen in the rat model, suggesting that intraductal administration of cytotoxic agents might eliminate the epithelial tissue lining the ducts.

DISCUSSION

Investigation of the role that the intraductal route may play in the treatment and prevention of breast cancer is in its infancy. It has been shown that paclitaxel prevents tumor growth in the MNU-induced rat breast cancer model (7). We have demonstrated that intraductal PLD is associated with a potent protective effect both in the MNU-induced rat model and in the spontaneous Her2/*neu* transgenic mouse model (3). We have extended our analysis to four additional agents in this study to show that intraductal 5-FU, carboplatin, and PLD all provide benefit in the same rat model, whereas methotrexate and nab-paclitaxel do not. Administration of intraductal 5-FU, a key component of first-line regimens for breast cancer treatment, was associated with a significant protective effect against early lesions in rats, as well as complete regression of most established tumors.

We did not observe major systemic side effects in rats that were treated with the equivalent (5-FU) or higher doses of a clinical human dose (PLD, carboplatin, methotrexate, or nab-paclitaxel). Treatment with 5-FU was associated with the greatest antitumor effects among the five agents, as evidenced by the lowest number of neoplastic events and a low Ki-67 labeling index. The observed transient hair thinning in rats in response to intraductal 5-FU indicates that a systemic concentration is achieved, which may be explained by the agent's small molecular weight and short half-life. Further, intraductal 5-FU administered to four ducts resulted in protection of the remaining eight uninjected mammary glands from tumor

development (72% tumor-free mammary glands), and with greater efficacy than that seen in rats after intravenous administration of 5-FU (51% tumor-free mammary glands). Thus, intraductal 5-FU was superior to intravenous 5-FU in preventing mammary tumorigenesis in the injected mammary glands and also showed efficacy in the uninjected glands in the same animal, implicating the participation of a systemic factor in tumor prevention with this particular agent.

This finding is in line with previous reports of potentiation of antitumor immune response using cyclophosphamide and doxorubicin in patients with breast cancer (8) and using gemcitabine and 5-FU in colon cancer (9). The observed occasional PLD-associated skin erosion close to the rats' teats might be from an overflow of drug when administered to smaller-size mammary glands that are closer to the neck and tail (glands 1 and 6) compared to the larger abdominal and inguinal glands. Similarly, we observed nipple irritation in the clinical trial, which we attributed to the drug's effect on the skin surface (PLD is a known irritant). By histopathological analysis, epithelial cell destruction was confirmed only in the PLD-treated animals. Because PLD is a slow-release formulation, it can remain in the mammary gland for a long time compared to intravenous PLD-injected rats (3). Consistent with the preclinical studies, we observed epithelial cell loss in H&E sections of mastectomy specimens obtained in the clinical trial (Fig. 2, C and D).

Intraductal carboplatin was associated with beneficial effects for treatment both in early lesions and in late established mammary carcinomas. Carboplatin is a platinum complex and active in a wide range of solid tumors (10). Although carboplatin has not been in common use against breast cancer since the introduction of anthracyclines and taxanes, several studies suggest that it is associated with therapeutic effects in combination with other agents, or in specific subtypes of breast cancer such as in women who are *BRCA1* or *BRCA2* mutation carriers (11). Thus, carboplatin may warrant further investigation, in particular in *BRCA* mutation carriers. Although treatment with methotrexate or nab-paclitaxel was associated with few side effects, we did not observe a statistically significant reduction in Ki-67 labeling index in the treated mammary glands (table S3) or in tumor incidence compared to untreated controls (Table 1) in the rat model, suggesting that those agents might not be appropriate for intraductal treatment in humans.

Our preclinical studies show that carboplatin, 5-FU, and PLD are suitable for further intraductal testing in clinical trials. At the same time, our clinical trial data with PLD support the feasibility of intraductal administration of agents. We demonstrated that administering PLD into one duct of women awaiting a mastectomy was safe and associated with minimal side effects. Nevertheless, our clinical trial is associated with several limitations and should be regarded as a proof-of-principle study. First, we evaluated only a few dose levels in a small number of women ($n = 17$). Second, we administered PLD once into one ductal system. Safety should be investigated with longer observation period in women who are awaiting mastectomy and lumpectomy or those at high risk who are considering prevention options.

Preliminary results have been presented from a study in 15 women awaiting mastectomy who received either PLD or carboplatin intraductally to five to eight ducts at three dose levels before the planned surgery (12). As in our study, Love *et al.* reported that intraductal administration was associated with only mild breast discomfort during drug delivery (12). A review of mastectomy specimens revealed that the drugs were distributed widely throughout the ductal systems and reached the terminal duct lobular units. These results, together with our report, support the ability to administer cytotoxic drugs into breast ducts with minimal toxicity.

The 2-mg dose of intraductal PLD was a reasonable starting point in our study because we did not observe harmful effects in rats at an equivalent dose. Intraductal PLD administration was associated with a dose-dependent increase in both systemic and local exposure to doxorubicin and the metabolite, yet with considerably lower systemic concentrations than intravenous dosing. We did not detect doxorubicin or its metabolite, doxorubicinol, in contralateral (untreated) breast tissue. We note that although we include models and patients with established tumors in the studies described here, because treatment of breast cancer requires both local and systemic therapy, we believe that intraductal administration of agents is applicable in the preneoplasia and prevention settings.

Great care needs to be exercised in the choice of agents that may provide long-term benefit in the prevention setting. Although the cytotoxic agents we used appear effective in animal models, their potential carcinogenic properties and differential metabolism across species must be recognized. For instance, patients treated with systemic doses of antimetabolites, such as 5-FU and methotrexate, have experienced a lower rate of iatrogenic cancers than those with DNA intercalating agents, such as doxorubicin (13). Natural products, such as curcumin or vitamin D, might be safer alternatives for therapy and prevention of early breast cancer.

MATERIALS AND METHODS

Animal studies and intraductal injection

Female Sprague-Dawley rats, 3 to 4 weeks of age, were housed in a controlled environment with a 12-hour light/dark cycle for at least 7 days before undergoing experimental procedures. Rats received a single intraperitoneal injection of MNU (50 mg/kg) (Sigma-Aldrich) between 4 and 5 weeks of age and allowed access to standard laboratory food and water ad libitum. Animal experiments were performed with approval and guidelines of the Animal Care and Use Committee of Johns Hopkins University School of Medicine.

Rats were anesthetized by isoflurane/oxygen inhalation. Keratin plugs were removed from the surface of the teat by rubbing gently with gauze soaked in alcohol, revealing the duct orifice. Mammary ducts were cannulated with a 34-gauge, blunt-ended needle attached to a syringe. Drug (100 μl per teat) was infused slowly into the mammary gland while the opening was visualized under a dissection microscope. Agents included 5-FU, carboplatin, methotrexate, nab-paclitaxel (Abraxane, provided by Abraxis Inc.), and PLD (Doxil: doxorubicin-HCl liposomal injection). The human dose equivalent was calculated with body surface area of 100-g weight of rat with the following formula: $(k \times w^{2/3}) \times 10^{-4}$, where k is a constant (9.5 $m^2/g^{2/3}$) and w is the weight of the rat (in grams) (14).

Treatment of early lesions in rats

To evaluate the effect of the drugs administered at doses higher than the safe human equivalent, effectiveness in reducing the number of occult MINs and microscopic tumors, and to perform whole mounts and histopathological examination, we treated rats ($n = 5$, 60 mammary glands) 14 days after MNU exposure with a single intraductal injection of 5-FU (12 mg per rat), carboplatin (6 mg per rat), methotrexate (4 mg per rat), nab-paclitaxel (60 mg per rat), PLD (0.96 mg per rat), or saline (vehicle) to all 12 mammary glands, or left them untreated (none), and killed them 7 weeks later. Mammary glands were evaluated for the presence of tumors, both macroscopically and by histology with whole mounts and H&E staining.

In a second set of rats ($n = 5$ per treatment group), the agents carboplatin (6 mg per rat), methotrexate at two doses (4 or 10 mg per rat), and nab-paclitaxel (60 mg per rat) were

administered intraductally once, 14 days after MNU injection, to all 12 glands of each rat (60 glands per agent). Treated and untreated rats were observed for 22 weeks.

Treatment with 5-FU was initiated 14 days after MNU injection and was administered once weekly for 4 weeks intravenously or intraductally to four teats (second to the fifth) on the right side of the rat. Treated (n = 11) and untreated (n = 10) rats were observed for 22 weeks after the initiation of treatment and palpated weekly for the presence of tumors; intraductal 5-FU–treated rats were palpated at weekly intervals for the presence of tumors in both injected (n = 44, four glands per rat) and uninjected (n = 88, eight glands per rat) glands.

Treatment of established tumors in rats

To test the therapeutic effects of intraductal 5-FU, we treated rats with MNU and palpated them weekly for mammary tumors. When a mammary tumor was palpable (125 to 200 mm^3), the rats (n = 28, 14 in each group) were randomized to treatment with intraductal or intravenous 5-FU (1 mg) weekly for 4 weeks. The largest diameter (a) and the shortest dimension (b)—perpendicular to a—of the tumor were measured weekly. The tumor mass was calculated with the following formula: tumor mass (in mm^3) = (ab^2)/2 (15).

Histopathology

Mammary glands were dissected and whole mounts and paraffin-embedded sections were prepared as described previously (16). Briefly, for whole mount, mammary glands were removed and spread flat on slides and then fixed in 10% neutral buffered formalin for 24 hours. After fixation, glands were defatted in acetone for 12 hours, stained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and visualized by light microscopy. For paraffin-embedded sections, a small piece of mammary gland was fixed for 24 hours in 10% neutral buffered formalin. Sections and staining were performed according to standard procedures by the Surgical Pathology Laboratory at the Johns Hopkins Hospital.

Immunostaining was performed with the Vectastain ABC Elite Kit (Vector Laboratories) with monoclonal anti-Ki-67 antibody (BD Biosciences). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. Deparaffinized sections were placed in antigen unmasking solution (Vector Laboratories) and heated in a household microwave oven at 99°C for 20 min. Sections were cooled to room temperature, and endogenous peroxidase activity was quenched by immersing sections in 0.3% hydrogen peroxide for 30 min. Tissue sections were blocked with 5% normal horse serum diluted in phosphate-buffered saline at room temperature for 20 min. Tissues were then incubated in primary antibody (1:100) at 4°C overnight. Tissue sections were washed and incubated with respective secondary antibody (1:1000) for 30 min at room temperature. After washing, slides were incubated in the ABC complex for 30 min at room temperature and then stained with 3,3′-diaminobenzidine (Vector Laboratories) until optimal staining was obtained. Sections were counterstained with hematoxylin and mounted.

The immunostained epithelial cells of mammary ducts smaller than 100 μm in diameter were counted in randomly selected 40× objective magnification fields for each sample and scored on a total of at least 5000 nuclei per case. Ki-67 labeling index was calculated as follows: (number of positive cells) × 100/(total number of epithelial cells). The estimate of cell counts was analyzed by an observer who was blinded to the treatment groups.

Clinical trial patient population and procedures

The phase 1 feasibility and safety, dose-escalation, single-institution study was approved by the Johns Hopkins Institutional Review Board (http://www.clinicaltrials.gov,

NCT00290732). Details of patient accrual and omission are described in the Supplementary Material.

Statistical analysis

Animal studies—Tumor-free survival of glands was defined as the time from MNU injection to early lesion development in each gland or to animal death. Glands of the animals that were euthanized were censored as of the last date of tumor assessment. Survival distributions were graphically displayed with Kaplan-Meier curves. Hazard ratios were estimated with 95% confidence interval (CI) with the Cox proportional hazards model, where correlations between within-cluster (animal) observations were taken into account. Means of Ki-67 labeling index were compared between treatment groups with analysis of variance (ANOVA). A mixed-effects model for repeated-measures data was used to evaluate the treatment effects on established tumors, where the interaction effect between treatment and time was tested. Multiple comparisons were adjusted with standard procedures. All tests were two-sided and considered statistically significant at $P < 0.05$. Analyses were performed with STATA (version 8.2) and SAS (version 9.2) software.

Clinical studies—The primary objective of the phase 1 study was to evaluate the feasibility, safety, and maximum tolerated dose (MTD) of administering PLD into one duct of women with breast cancer awaiting mastectomy. The study was conducted with a standard 3 + 3 dose-escalation design. Dose-limiting toxicity (DLT) was defined as grade 3 to 4 hematological toxicity or grade 2 to 4 nonhematological toxicity of pain or extravasation changes in the ipsilateral breast, or palmar-plantar erythrodysesthesia, with CTCAE version 3.0. In addition, grade 2 to 4 local skin toxicity was considered DLT.

Three women were enrolled to a dextrose infusion (dose level 0) to determine the feasibility of study logistics and local adverse events with the drug vehicle alone. The study would proceed to dose level 1 if no grade 2 (moderate) or higher pain was reported in the treated breast and the feasibility of the study requirements was confirmed. The dose levels tested included 2, 5, and 10 mg of PLD per duct corresponding to levels 1, 2, and 3 (Table 3). Three to six women were enrolled in successive cohorts, and standard dose-escalation rules were used. Dose was to be escalated until the DLT was observed and the MTD was defined. Further escalation above 10 mg (dose level 3) was not planned. If no DLT was observed at 10 mg, it would be determined that MTD was not reached in this study, and we would consider reformulating PLD for increased doses, PLD treatment using multiple ducts, or using other agents in follow-up studies. We added a control cohort that consisted of three women treated with intravenous PLD to compare pharmacokinetic parameters between intraductal- and intravenous-treated women.

Adverse event laboratory tests were summarized with descriptive statistics. Doxorubicin and doxorubicinol concentrations were summarized by dose level with descriptive statistics. All patients who received any amount of intraductal PLD were evaluated for toxicity. All analyses were performed and all conclusions were based on eligible patients who initiated the protocol treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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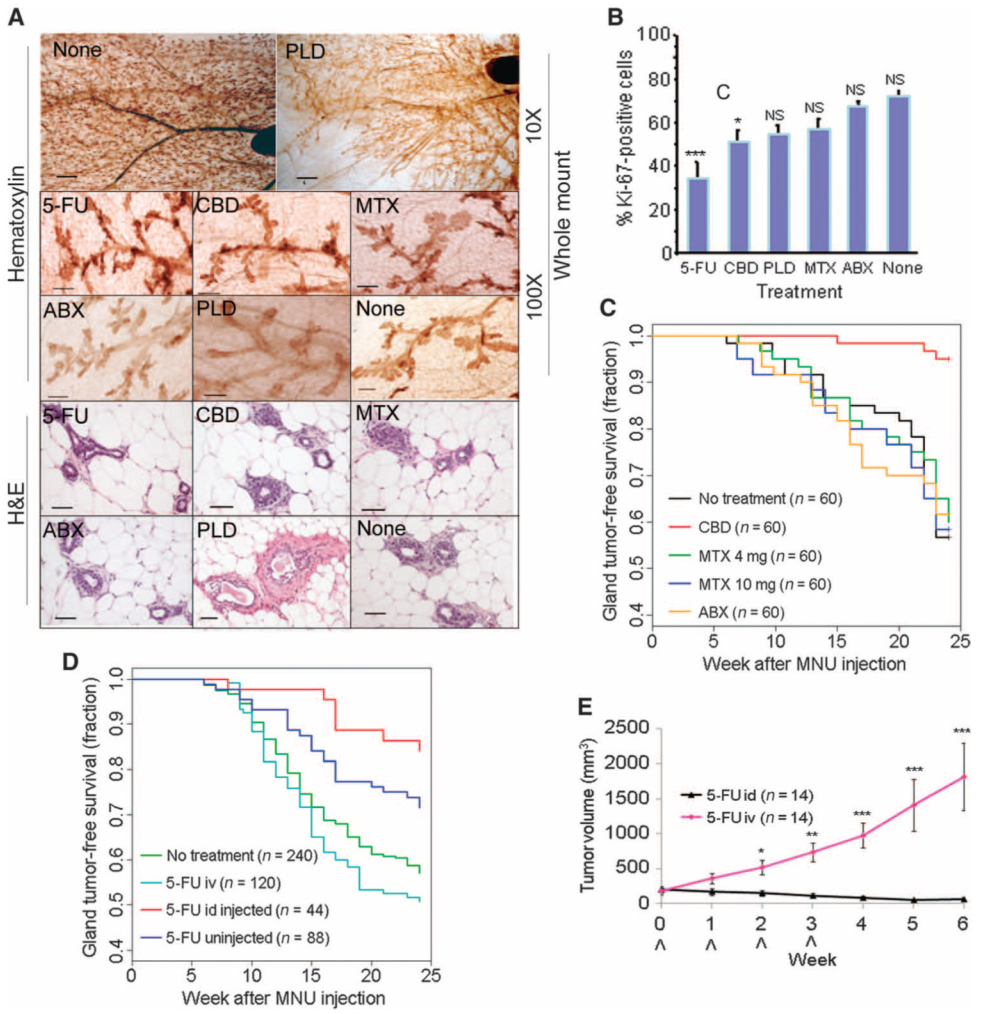

Fig. 1.

Preclinical analysis of intraductal therapy of MNU-induced rat mammary tumors. (**A**) Ductal branching structure density in representative hematoxylin-stained whole mounts (top panel, ×10 magnification) of the fourth mammary gland of an uninjected rat (None) and an intraductal PLD-injected rat 7 weeks after treatment. Examination at higher power (100) of the whole mount of the mammary gland after intraductal treatment with 5-FU, carboplatin (CBD), methotrexate (MTX), nab-paclitaxel (ABX), PLD, or None. Bottom panel, H&E-stained sections of formalin-fixed, paraffin-embedded mammary glands after treatment with 5-FU, carboplatin, methotrexate, nab-paclitaxel, PLD, or None. Top panel scale bars, 2 mm (×10) or 200 μm (×100). H&E scale bars, 50 μm. (**B**) Percentage of Ki-67–positive cells after intraductal drug or saline treatment. Data are averages ± SEM ($n = 5$ rats per treatment group). *$P < 0.05$; ***$P < 0.0001$ by ANOVA with multiple comparisons adjusted with Tukey's procedure. (**C**) Mammary gland tumor-free survival in response to intraductal chemotherapy. Female rats received MNU (week 0), and drug was administered intraductally 2 weeks later. Rats were killed 22 weeks after start of treatment. (**D**) Mammary gland tumor-free survival in response to intraductal 5-FU therapy. Female rats received MNU (week 0); 2 weeks later, the rats were left untreated or treated with 5-FU intravenously or intraductally. In each rat, only four mammary glands received 5-FU, whereas eight mammary glands remained untreated. Rats were killed 22 weeks after start of treatment. (**E**) Growth of MNU-induced mammary tumors (in mm^3) over 6 weeks after

treatment of a single palpable tumor with either intraductal or intravenous 5-FU. Rats received treatment four times at weekly intervals. Data are means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, using a mixed-effects model.

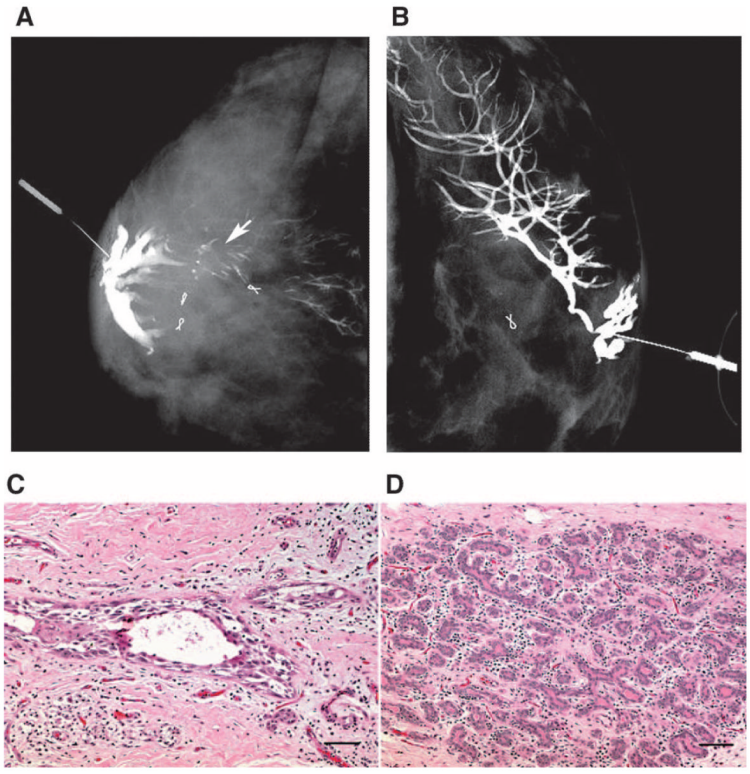

Fig. 2.

Representative ductogram and histopathology images from the clinical trial. (**A**) A ductogram from a 29-year-old participant, which outlines dilated ducts surrounding the area of the tumor, located in the upper aspect of the right breast (arrow). (**B**) A ductogram from a 37-year-old participant. The ductogram outlines a normal branching duct coursing superior to the site of malignant calcifications in the tail of the left breast. (**C**) A histological specimen of a prophylactic mastectomy representing a treated lobule (blue-dyed region). (**D**) Histological specimen of the same prophylactic mastectomy shown in (C) of a non-treated region (non-blue-dyed region). Scale bars, 100 μm.

Table 1

Therapeutic effect of intraductally administered cytotoxic agents on MNU-induced rat mammary tumors. Two weeks after MNU injection, when hyperplasias, MINs, and microscopic carcinomas—but no palpable lesions—were present in the mammary glands, rats were left untreated or were treated with a single intraductal injection of one of the five drugs to all 12 glands. Rats were killed at 22 weeks after the initiation of drug treatment. Time to emergence of tumors was analyzed with the individual gland as the analysis of unit with Cox proportional hazards model, where correlations between observations within the same cluster (rat) were taken into account. Multiple comparisons were adjusted with the Bonferroni procedure. CI, confidence interval; CBD, carboplatin; MTX, methotrexate; ABX, nab-paclitaxel.

Treatment comparison	Number of tumors at the end of the study per 60 glands	Hazard ratio	95% CI	*P*
No treatment versus CBD	26 versus 3	10.4	4.83–22.6	<0.0001
MTX (4 mg) versus CBD	24 versus 3	9.62	4.06–22.8	<0.0001
MTX (10 mg) versus CBD	25 versus 3	10.5	4.09–27.0	<0.0001
ABX versus CBD	23 versus 3	9.70	4.26–22.1	<0.0001
No treatment versus MTX (4 mg)	26 versus 24	1.09	0.67–1.76	>0.999
No treatment versus MTX (10 mg)	26 versus 25	0.99	0.53–1.85	>0.999
No treatment versus ABX	26 versus 23	1.08	0.71–1.64	>0.999
MTX (10 mg) versus MTX (4 mg)	25 versus 24	1.09	0.53–2.27	>0.999
MTX (10 mg) versus ABX	25 versus 23	1.08	0.55–2.16	>0.999
ABX versus MTX (4 mg)	23 versus 24	1.01	0.57–1.78	>0.999

Table 2

Therapeutic effect of intraductally administered 5-FU on MNU-induced rat mammary tumors. Two weeks after MNU injection, when hyperplasias, MINs, and microscopic carcinomas—but no palpable lesions—were present in the mammary glands, rats were treated with the indicated agent. Eleven rats received 5-FU injected intraductally into 4 mammary glands (second to the fifth) each (n = 44 mammary glands total), whereas the remaining 8 mammary glands in each animal received no treatment (n = 88 total; labeled as "uninjected"). Ten rats received 5-FU intravenously (120 mammary glands total) and 20 rats remained untreated (240 mammary glands total). Rats were killed at 22 weeks after the initiation of treatment. Time to emergence of tumors was analyzed with the individual gland as the analysis unit with Cox proportional hazards model, where correlations between observations within the same cluster (rat) were taken into account. Multiple comparisons were adjusted with the Bonferroni procedure. id, intraductal; iv, intravenous.

Treatment comparison	Number of tumors at the end of the study/number of glands	Hazard ratio	95% CI	*P*
No treatment versus 5-FU id injected	103/240 versus 7/44	3.30	1.50–7.25	0.018
5-FU iv versus 5-FU id injected	59/120 versus 7/44	4.02	1.73–9.36	0.006
5-FU iv versus 5-FU id uninjected	59/120 versus 25/88	2.06	1.19–3.55	0.054
No treatment versus 5-FU id uninjected	103/240 versus 25/88	1.69	1.08–2.64	0.126
5-FU id uninjected versus 5-FU id injected	25/88 versus 7/44	1.95	0.88–4.36	0.612
No treatment versus 5-FU iv	103/240 versus 59/120	0.82	0.55–1.22	>0.999

Table 3

Intraductal protocol for clinical trial. Three women were enrolled in a vehicle-only cohort (dose level 0) to establish study logistics and local adverse events with the vehicle alone. The dose levels tested included 2, 5, and 10 mg of PLD per duct corresponding to levels 1, 2, and 3. The number of attempted procedures and adverse events is summarized.

Dose level	PLD dose (mg), in 5 ml of dextrose	Ductal cannulation (attempted/successful)	Adverse events (mild)	
			Breast fullness	Breast or nipple pain/discomfort
0	0	3/3	3/3	2/3
1	2	4/3 *	3/3	3/3
2	5	4/3 †	2/3	3/3
3	10	6/6	1/6	6/6
Total		17/15	9/15 (60%)	14/15 (93%)

* One duct was cannulated successfully; however, PLD could not be instilled, likely owing to extensive disease (4.5-cm tumor, grade III, with one of three positive lymph nodes).

† Cannulation was not successful in bilateral breasts, likely owing to previous excisional biopsies.

Table 4

Doxorubicin and doxorubicinol concentrations in plasma and breast tissue. A value of "0" was noted for concentrations below the limits of buantification of the analytical assay. NA, not applicable; NC, specimen not collected. id, intraductal;.iv, intravenous.

PLD dose (mg), route of administration	Subject	Days to surgery	Doxorubicin/doxorubicinol (nM) in plasma					Doxorubicin/doxorubicinol (nmol/g) in breast tissue		
			0 hours	4 hours	24 hours	8 days	Day of surgery	Blue-dyed region	Non-blue-dyed region	Contralateral breast*
0, id (dextrose)	001	5	NC	NC	NC	NC	NC	0/0	0/0	NC
	002	5	NC	NC	NC	NC	NC	0/0	0/0	NC
	003	23	NC	NC	NC	NC	NC	0/0	0/0	NC
2, id	101	17	0/0	0/0	0/0	0/0	0/0	0/0	0/0	NC
	102	13	0/0	28.5/0	0/0	0/0	0/0	0/0	0/0	0/0
	104	40	0/0	0/0	0/0	NC	0/0	0/0	0/0	0/0
5, id	202	20	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	203	12	0/0	18.3/0	0/0	0/0	0/0	0/0	0/0	0/0
	204	10	0/0	0/0	0/0	0/0	0/0	1.73/0.09	0/0	0/0
10, id	301	9	0/0	0/0	0/0	0/0	0/0	0.42/0	4.58/0.21	NC
	302	12	0/0	132.0/0	481.0/22.6	134.0/0	11.3/0	0.07/0	0.07/0	0/0
	303	7	0/0	24.4/0	480.0/22.6	NC	902.0/36.9	1.23/0.05	1.00/0.04	0/0
	304	18	0/0	0/0	0/0	0/0	0/0	0.11/0	0/0	NC
	305	13	0/0	0/0	60.9/0	0/0	0/0	0.24/0	10.82/5.26	NC
	306	18	0/0	0/0	87.3/0	346/17.8	54.5/0	2.86/0.16	0.71/0.04	NC
68, iv†	401	8	0/0	65,900/8,090	79,600/3,190	NC	28,400/869	NA	NA	0/0
80, iv†	402	13	0/0	55,100/4,000	33,700/1,980	2,760/294	163/11	NA	NA	0/0
68, iv†	403	21	0/0	41,300/2,260	44,400/2,480	18,300/286	228/16	NA	NA	0.21/0

*Specimens were obtained for baseline doxorubicin measures as a control and because of previous doxorubicin exposure in some women.

†40 mg/m2.