Ductal Access for Prevention and Therapy of Mammary Tumors

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Abstract

In cancer patients and in those at high risk, systemic exposure to agents for therapy or prevention is accompanied by undesirable side effects. We hypothesized that it is possible to prevent and treat breast cancer by introducing anticancer agents into the mammary ductal network. Here, we show the efficacy of intraductally administered anticancer agents 4-hydroxytamoxifen and pegylated liposomal doxorubicin (PLD) in the prevention and treatment of breast cancer using the rat *N*-methyl-*N*′-nitrosourea–induced and spontaneous HER-2/*neu* transgenic mouse (neu-N) models of breast cancer. Intraductal administration of PLD to neu-N mice caused regression of established tumors and prevented tumor development more effectively than i.v. injection ($P < 0.0001$). Intraductal administration resulted in lower circulating levels of PLD compared with i.v. administration, with no evidence of systemic toxicity or long-term histopathologic changes in the mammary gland. Compared with systemic administration, intraductal injection provides direct access to breast lesions with higher local and lower systemic drug exposure. These studies suggest that this approach has potential for application to prevention and neoadjuvant therapy of early breast cancer. (Cancer Res 2006; 66(2): 638-45)

Introduction

Currently, cancer chemotherapeutics are administered predominantly through the systemic route orally or by i.v. injection. Although systemic administration is the most efficient route of delivery to cancers in many organs, it also exposes all healthy tissues to the delivered drugs, frequently resulting in harmful side effects. The human breast provides an alternate route for tumor access, the mammary ductal networks that terminate at openings at the nipple. More than 95% of breast cancers arise from the epithelial cells making up the gland lobules and ductal networks. Thus, it is reasonable to speculate that intraductal administration of anticancer agents might provide more direct access to preneoplastic lesions and tumors while limiting systemic exposure and reducing adverse side effects. In this study, we tested the ability of intraductal administered anticancer agents 4-hydroxytamoxifen (4-OHT) and pegylated liposomal doxorubicin (PLD) to prevent and treat breast cancer using the rat *N*-methyl-*N*′-nitrosourea (MNU)–induced and neu-N transgenic mouse models of breast cancer.

The approach of introducing reagents through the teat for the purposes of tumor induction, prevention, and therapy has precedence. McFarlin and Gould studied the role of activated Raf in mammary carcinogenesis by infusing mammary glands of rats with retroviral vectors to express the protein in a small number of mammary cells *in situ* (1). To test the idea that reducing the number of proliferating cells in mammary gland lobules of MNU-treated rats will reduce tumorigenesis, Sivaraman et al. injected adenoviral vectors carrying the thymidine kinase gene into the mammary duct followed by i.p. administered gancyclovir treatment. Very efficient expression of thymidine kinase protein and ablation of proliferating cells was achieved. However, contrary to expectation, the number of mammary tumors in the treated group was higher (2). In another study, using the concept of intraductal therapy, MNU-induced mammary tumors were treated either through the duct or i.p. with the microtubule inhibitor paclitaxel (3). The incidence of mammary carcinoma was significantly reduced in rats treated intraductally, accompanied by an increase in apoptosis and a reduction in microvessel density, compared with tumors in the rats administered paclitaxel by the i.p route. They concluded that local administration of paclitaxel may be useful for treatment of breast cancer.

In this article, we provide proof of principle showing the effectiveness of intraductally administered 4-OHT and PLD in the prevention and therapy of rat mammary tumors using the well-known chemical carcinogen, MNU. In addition, the successful applicability of the approach in prevention and therapy and toxicity variables are addressed in the spontaneously developing HER-2/*neu* murine mammary tumor model, neu-N. The advantages and the clinical translational potential of this route of injection for prevention and therapy of breast cancer are discussed.

Materials and Methods

Intraductal injection. Mice or rats were anesthetized by isoflurane/oxygen inhalation. Keratin plugs were removed from the surface of the nipple by rubbing gently with gauze soaked in alcohol, revealing the duct orifice. Mammary ducts were cannulated using a 1.0-cm, 34-gauge, blunt-ended needle (Hamilton, 90083) attached to a 1-mL tuberculin syringe. Drug or PBS (50 μL for mice and 100 μL for rats) was infused into the mammary gland while visualizing the opening under a dissection microscope.

Histopathology. Mammary glands were sharply dissected, and either whole mounts or paraffin-embedded sections were prepared. For whole mounts, mammary glands were prepared as previously described (4). Briefly, mammary glands were removed, fixed in methanol/chloroform/glacial acetic acid, and flattened for 24 hours between glass plates. Glands were then placed in a tissue cassette and fixed in methanol/chloroform/glacial acetic acid for an additional 24 hours. Following fixation, glands were defatted in acetone for 48 hours, stained with iron hematoxylin, dehydrated in graded ethanol, cleared in CitriSolv (Fisher Scientific, Hampton, NH), and

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visualized by light microscopy. For paraffin-embedded sections, mammary glands were fixed for 24 hours in 10% neutral buffered formalin. Generation of paraffin-embedded sections and H&E staining were done according to standard procedures by the Surgical Pathology Laboratory at the Johns Hopkins Hospital.

Quantitation of doxorubicin in rat plasma. High-pressure liquid chromatography (HPLC) with fluorescence detection was used to measure total doxorubicin concentrations in rat plasma. Briefly, blood was collected, and plasma samples were extracted in 1:4 isopropyl alcohol/chloroform, and the organic layer was evaporated to dryness under a stream of nitrogen gas. Samples were subsequently reconstituted in a solution of acetonitrile/methanol/water (4:3:3), and the concentration of doxorubicin was determined using a Waters Alliance HPLC system equipped with a fluorescence detector (Waters Corp., Milford, MA) at an excitation wavelength of 490 nm and emission wavelength of 580 nm.

Statistical analysis. Prevention of tumor development in Sprague-Dawley rats with intraductal administration was evaluated using the generalized estimating equations method, assuming an exchangeable covariance structure of the correlated measurements that were taken on the same rat. Gland tumor-free survival was defined as time to tumor that occurred on each gland or time to sacrifice. Mouse tumor-free survival was defined as time to tumor that occurred in first gland of the mouse or time to sacrifice after initiation of treatment. Survival curves were graphically displayed using the Kaplan-Meier method. The Cox proportional hazards model was applied to simultaneously account for the treatment effects, treatments on the other side and gland when appropriate, where the correlation among measurements taken on the same mouse was taken into account.

The longitudinal data of tumor growth were analyzed using a mixed-effects model, in which an exchangeable covariance structure was assumed by accounting for the correlated measurements taken on the glands from the same mouse. The initial tumor size was stratified by <25, 25 to 100, and >100 mm^2. Quadratic models were applied to the data when a nonlinear trajectory was identified, and treatment effects were compared on the mean tumor size at selected time points. Analyses were conducted using STATA software (version 8.2) and SAS System software (version 9.1). All statistical tests were two sided and were considered statistically significant at $P < 0.05$.

Results

Intraductal Injection Affords Access to the Entire Mammary Gland

Unlike the human breast, which is composed of many ductal systems, each rat and mouse breast is composed of one ductal tree (consisting of a network) that terminates at the teat. We first tested our ability to access the entire rat and mouse mammary gland through the teat by injecting the dye crystal violet (1.68%, Sigma-Aldrich, St. Louis, MO). Following intraductal injection, the mammary gland was removed and examined by whole mount. The crystal violet dye traveled to every portion of the mammary gland, including the most distal portion of the ductal network, the terminal end buds, where most breast cancers are believed to arise (Fig. 1*A*).

Model I: MNU-induced Rat Mammary Tumor Model

Intraductal 4-OHT Is as effective as s.c. tamoxifen in preventing MNU-induced mammary tumorigenesis. Systemically delivered tamoxifen has emerged as a highly effective anticancer agent in the treatment of early cancer and more recently in the prevention setting (5). As proof of concept, we tested the tumor preventive effects of tamoxifen delivered by the intraductal route in the estrogen receptor–positive, MNU-induced rat mammary tumor model. First, in a time course experiment, by examination of iron hematoxylin-stained whole-mount preparations of mammary glands from MNU-treated rats at two weekly intervals, we confirmed previously published observations that Sprague-Dawley rats develop preneoplastic lesions like atypical ductal hyperplasias and ductal carcinoma *in situ* (DCIS) within 14 to 21 days (in 12 of 12 mammary glands examined) following MNU injection (6). This was followed by the appearance of mammary carcinomas within 3 to 4 months.

Having confirmed that by 21 days following MNU treatment all the mammary glands of rats harbored preneoplasias, we tested the chemopreventive effects of 4-OHT and tamoxifen in this model system. On day 21 after MNU exposure, rats were administered either intraductal injections of 4-OHT (50 μg), tamoxifen (50 μg), or oil; s.c. injections of tamoxifen (50 μg) or oil; or no treatment. Tamoxifen intraductal had no protective effect (17 tumors/98 glands; Table 1), likely due to the fact that active metabolites of tamoxifen, including 4-OHT, are produced by liver enzymes (7), and such activation does not occur in the mammary gland. On the other hand, intraductal injections of 4-OHT (8–11), starting on day 21, prevented the development of tumors (1 of 201 glands) compared with untreated animals (62 of 288 glands; $P < 0.0001$). This protective effect was comparable with s.c. administered tamoxifen in preventing tumorigenesis (0 of 144 glands), whereas groups that received oil intraductal developed a similar number of tumors (17 of 72) as the untreated group. Thus, in this model, the active metabolite of tamoxifen, 4-OHT, was as effective, given intraductally as tamoxifen when administered s.c. in preventing mammary carcinogenesis.

Pharmacology and effectiveness of PLD in therapy and prevention of rat mammary tumorigenesis. Next, we tested the ability of the anticancer agent, pegylated liposomal doxorubicin (PLD, Johnson and Johnson, Bridgewater, NJ), to treat and prevent breast cancer when administered by intraductal injection. PLD has been shown to have a longer serum half-life than free doxorubicin as well as reduced toxicity (12–14).

Clearance and toxicity of intraductal PLD in Sprague-Dawley rats. To determine the distribution of the drug upon intraductal injection, Sprague-Dawley rats were administered 400 μg PLD either by intraductal (100 μg/duct) or i.v. injection. Plasma was analyzed for doxorubicin concentration in blood samples taken 0, 4, 24, and 48 hours later by HPLC with fluorescence detection. Drug levels after intraductal injection peaked after 24 hours at 10.4 μmol/L, whereas levels following i.v. injection peaked after 4 hours at 103.7 μmol/L (Fig. 1*B*). No myelosuppressive effects were observed 1 week following intraductal administration of PLD as determined by bone marrow colony forming assay (ref. 15; data not shown). This supports the possibility that intraductal administration delivered more drug to the breast lesions while reducing the amount of drug reaching nontargeted tissues via the blood stream.

Therapeutic effects of intraductal PLD. For testing the therapeutic effects of PLD intraductally, 3- to 6-week-old rats were injected with MNU (50 mg/kg, i.p.), and ~500 mm^3 tumors were treated with 100 μg PLD (0.5 mg/kg) intraductally once a week for 2 weeks. After a period of 6 weeks, all animals that received no treatment were euthanized due to excessive tumor outgrowth. In stark contrast, 24 of 25 tumors treated with PLD by intraductal administration regressed completely (Fig. 1*C*). Animals remained tumor free during a 3-month follow-up period. These results suggest that intraductal administration provided adequate access for the successful treatment of established rat mammary tumors.

Preventive effects of intraductal PLD. To assess the effectiveness of PLD in preventing mammary tumorigenesis in rats, 3- to 6-week-old Sprague-Dawley rats were injected with MNU (50 mg/kg)

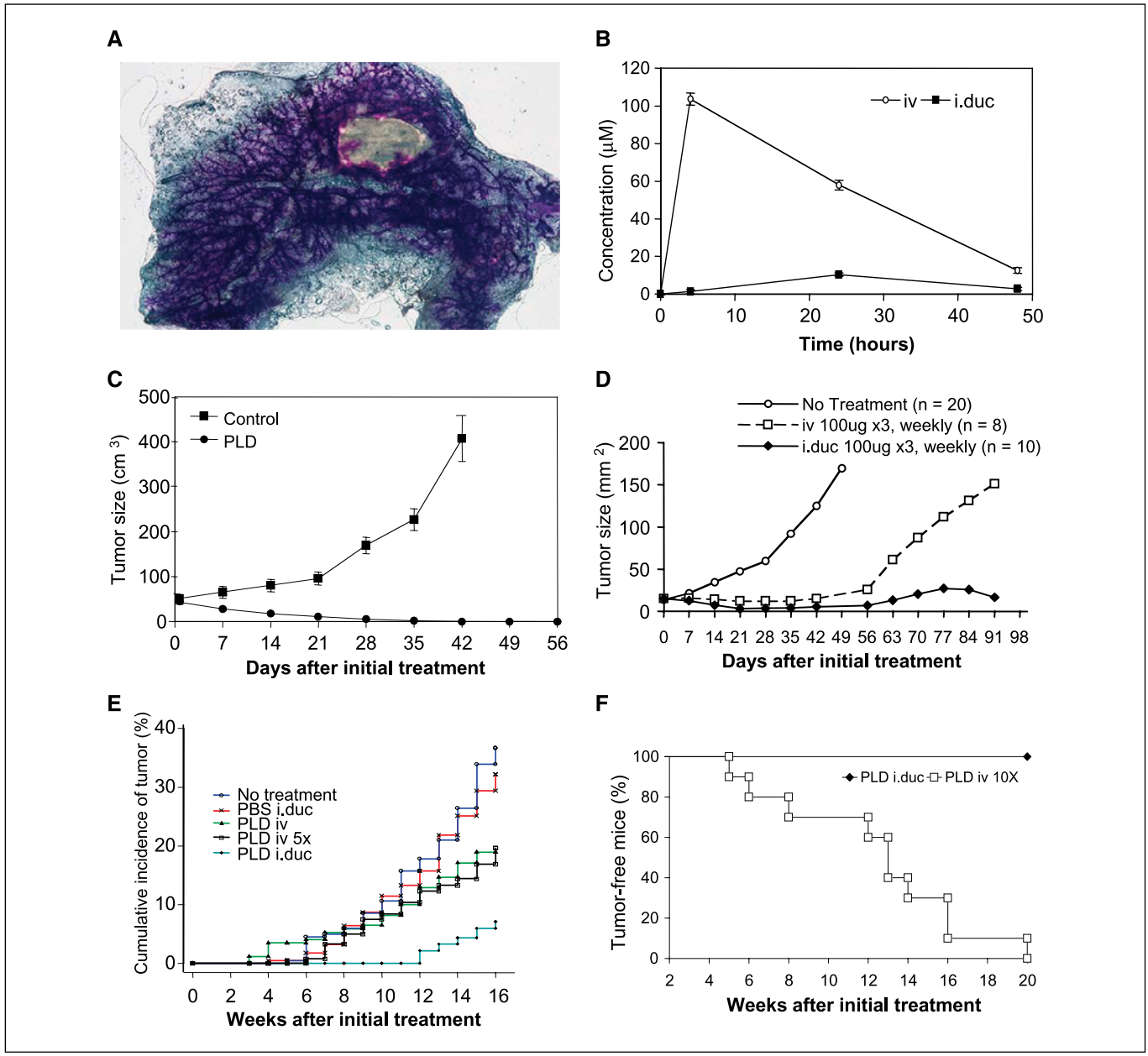

Figure 1. *A,* intraductal (*i.duc*) injection provides access to the entire mammary gland. Mouse mammary glands were injected with crystal violet by the intraductal method. Mammary glands were subsequently removed, whole mounts were prepared, and ducts infused with crystal violet were visualized by light microscopy (×10). *B,* intraductal administration results in lower systemic levels of doxorubicin relative to i.v. administration. Sprague-Dawley rats were administered a cumulative dose of 400 μg PLD by either intraductal or i.v. injection, and serum was analyzed for doxorubicin concentration. Representative of experiments done in duplicate. *Points,* mean doxorubicin concentration; *bars,* SD. *C,* intraductal administration of PLD results in complete regression of established rat breast tumors. Sprague-Dawley rats (n = 12) were administered MNU (50 mg/kg) to induce breast tumor development. Tumor treatment was initiated at an approximate size of 500 mm^3. Tumors were either administered PLD (100 μg) once per week for 2 weeks or received no treatment. Control (n = 27) and PLD-treated (n = 25) tumor sizes were recorded weekly. *Points,* mean tumor size over time; *bars,* SD. Representative of 24 of 25 tumors treated. *D,* intraductal administration of PLD achieves greater tumor regression than i.v. administration in neu-N mice. Mice bearing established breast tumors of <125 mm^3 were treated on day 0 by intraductal or i.v. administration of 100 μg PLD given three times over 3 weeks (×3, weekly), or were left untreated. Tumor sizes were recorded weekly. *Points,* mean tumor size (mm^3) over time. *E* and *F,* intraductal administration of PLD reduces the risk of breast tumor development in neu-N mice. *E,* 6-month-old, tumor-free neu-N mice received either PLD (40 μg) or PBS by intraductal or i.v. injection, or were left untreated. Intraductal injections were administered in one gland per week beginning with the inguinal gland in week 0 and ending with the ipsilateral cervical gland in week 4; each gland was treated only once. i.v. injections were administered either once in week 0 (*PLD iv*) or once per week for 5 weeks (*PLD iv 5*×). *F,* 6-month-old, tumor-free neu-N mice were administered PLD (40 μg) by intraductal or i.v. injection. Intraductal injections were administered to all glands of 10 mice beginning with the bilateral inguinal glands in week 0 and ending with the cervical glands in week 4. Treatment was repeated through weeks 6 to 10. I.v. injections were administered 1× weekly for 5 weeks (weeks 0-4), which were repeated from weeks 6 to 10. Palpable mammary tumors were measured using calipers. Time to tumor development (*E*) in each gland and (*F*) in each mouse as estimated using the Kaplan-Meier method.

i.p. On day 14, 100 μg PLD (0.5 mg/kg) was administered to each of four mammary glands per rat by intraductal injection, whereas the remaining glands received no treatment. Treatments were given once a week for 3 weeks, and animals were subsequently observed for 6 months. Tumor development was followed at weekly intervals. Tumors > 10 mm^3 and those that showed a progressive increase in size were scored. Intraductal administration of PLD significantly (P < 0.001, Table 2) protected animals against tumor formation

Table 1. Intraductal administration of 4-OHT prevents the development of breast tumors in Sprague-Dawley rats

Treatment	*n*	No. tumors/glands treated	Tumor-free glands (%)	95% Confidence interval	*P*
No treatment	24	62/288	78.5	73.3-83.1	—
4-OHT intraductal	20	1/201	99.5	97.3-100	<0.0001
Tamoxifen intraductal	10	17/98	82.7	73.7-89.6	0.289
Oil intraductal	6	7/50	86	73.3-94.2	0.162
Tamoxifen s.c.	12	0/144	100	97.9-100	<0.0001
Oil s.c.	6	17/72	76.4	64.9-85.6	0.643

NOTE: Female Sprague-Dawley rats were administered MNU (50 mg/kg) i.p. to induce tumor formation. Twenty-one days following MNU administration, rats were administered 4-OHT (50 μg), Tamoxifen (50 μg), oil, or no treatment by either by intraductal or s.c. injection. Treatments were administered once per week for 4 months, and tumor development was assessed over a period of 8 months. Treatment effects on the tumor development relative to no treatment were compared using the generalized estimating equations method, assuming an exchangeable covariance structure of the correlated measurements that were taken on the same rat. *n*, number of animals.

with only one tumor developing in 58 injected glands that received the full course of three injections. In contrast, 28 tumors developed in 120 noninjected glands of animals in the same group, an incidence similar to untreated animals, which developed 47 tumors in 240 glands. Thus, similar to our results with 4-OHT, the intraductal administration of PLD significantly prevented the development of rat mammary tumors.

Model II: Autochthonous Mammary Tumors in HER-2/neu Mice

Prevention and therapy with PLD through the intraductal route is more effective than i.v. in HER-2/*neu* transgenic mice. HER-2/*neu* transgenic mice (neu-N) develop *neu* overexpressing multifocal mammary adenocarcinoma, beginning at ~4 to 5 months of age. Fifty percent of neu-N mice develop tumors between 7 to 9 months after birth, and tumor incidence approaches 100% by 1 year (16).

Therapeutic effects of intraductal PLD. To test the efficacy of intraductal PLD administration in the treatment of established mammary tumors, tumors were treated by either intraductal or i.v. administration of PLD at various doses and schedules (Table 3). Treatment of tumors (<25, 25-100, or >100 mm^2) with PLD intraductally (40 μg) biweekly (once every other week) for 2 weeks significantly reduced the rate of tumor growth relative to treatment by i.v. administration. When the dose and schedule of PLD treatment was increased to 100 μg administered once weekly for 3 weeks, i.v. administration resulted in a predominantly cytostatic effect lasting for 56 days, after which tumors began to increase in size at a rate similar to untreated controls (Fig. 1*D*). In contrast, intraductal administration of PLD resulted in the complete remission of 8 of 10 tumors by 20 days. Although some tumors began to grow 56 days following initial treatment, the mean size of tumors was significantly smaller ($P = 0.015$), which became even more pronounced at 70 and 91 days ($P < 0.0001$) relative to tumors treated by i.v. administration (Table 3; Fig. 1*D*). Moreover, 4 of 10 tumors remained in complete regression during the entire 91-day follow-up period. Thus, intraductal administration of PLD showed a significantly greater efficacy in the treatment of established tumors relative to i.v. administration.

Preventive effects of intraductal PLD. Mammary tumors in the neu-N model also have the histologic appearance of DCIS at the early stages; however, they progress to become invasive at late stages and metastasize to the lungs after a long latency (17). To determine the ability of intraductal PLD administration to prevent the development of breast tumors in neu-N mice, 6-month-old post-breeder mice that had no palpable tumors were administered intraductal or i.v. injections of PLD (40 μg) or PBS, or were left untreated. intraductal injections were administered (n = 196 glands in 40 mice) in one gland per week beginning with the inguinal gland in week 0 and ending with the ipsilateral cervical gland in week 4. Thus, a total of five intraductal injections were done in each mouse in the same (right or left) set of glands, and each gland was administered a single intraductal injection of the agent. I.v. injections were administered either once (n = 170

Table 2. Intraductal administration of PLD prevents the development of breast tumors in Sprague-Dawley rats

Treatment	*n*	Tumors/Total no. glands	Tumor-free glands (%)	95% Confidence interval	*P*
No Treatment	20	47/240	80.4	74.8-85.2	—
PLD intraductal	15				—
Injected ducts		1/58	98.3	90.8-100	0.005
Uninjected ducts		28/120	76.7	68.1-83.9	0.271

NOTE: Female Sprague-Dawley rats were administered MNU (50 mg/kg) i.p. to induce tumor formation. Fourteen days following MNU administration, rats were administered 100 μg PLD by intraductal injection in each of 4 of 12 mammary glands. Treatments were administered once per week for 3 weeks, and tumor development was assessed over a period of 3.5 months. Statistical comparisons were the same as described in Table 1 using the generalized estimating equations method. *n*, number of animals.

Table 3. Intraductal administration of PLD reduces mean tumor size in neu-N mice relative to i.v. administration

Treatment comparison	Difference in mean tumor size (mm^2)	95% Confidence interval	*P*
<25 mm^2			
i.v. 40 μg ×2, biweekly vs no treatment	−66.8	−103.3 to −30.3	0.0004
intraductal 40 μg ×2, biweekly vs no treatment	−146.9	−181.3 to −112.5	<0.0001
intraductal 40 μg ×2, biweekly vs i.v. 40 μg ×2, biweekly	−80.0	−115.6 to −44.5	<0.0001
intraductal 100 μg ×2, biweekly vs intraductal 40 μg ×2, biweekly	−29.9	−59.6 to −0.297	0.048
i.v. 100 μg ×3, biweekly vs intraductal 100 μg ×2, biweekly	−4.36	−33.8 to 25.1	0.768
i.v. 100 μg ×3, weekly vs no treatment	−164.2	−196.4 to −131.9	<0.0001
intraductal 100 μg ×3, weekly vs no treatment	−198.4	−229.4 to −167.5	<0.0001
intraductal 100 μg ×3, weekly vs i.v. 100 μg ×3, weekly	−34.2	−61.5 to −6.98	0.015
25-100 mm^2			
i.v. 40 μg ×2, biweekly vs no treatment	−72.1	−114.5 to −29.6	0.001
intraductal 40 μg ×2, biweekly vs no treatment	−86.6	−127.5 to −45.7	<0.0001
Intraductal 40 μg ×2, biweekly vs i.v. 40 μg ×2, biweekly	−14.5	−64.8 to 35.8	0.564
>100 mm^2			
i.v. 40 μg ×2, biweekly vs no treatment	−177.9	−272.0 to −83.8	0.0004
intraductal 40 μg ×2, biweekly vs no treatment	−347.5	−441.6 to −253.4	<0.0001
intraductal 40 μg ×2, biweekly vs i.v. 40 μg ×2, biweekly	−169.6	−234.6 to −104.6	<0.0001

NOTE: neu-N mice bearing established mammary tumors of <25, 25 to 100, and >100 mm^2 were administered intraductal or i.v. injections of PLD (40 or 100 μg) or were left untreated. Two (×2) or three (×3) total injections were administered once per week (weekly) or once every other week (biweekly). Tumor sizes were recorded weekly and are reported at 7 weeks for tumors 25 to 100 and >100 mm^2 and 8 weeks for tumors <25 mm^2. *P* values and 95% confidence intervals were calculated for the difference in mean tumor among listed treatment comparisons using a mixed-effects model by assuming an exchangeable covariance structure of the correlated measurements taken on the same mouse.

glands in 17 mice), to control for the single treatment of each gland by intraductal injection, or once a week for 5 weeks (n = 120 glands in 12 mice), to control for the cumulative dose of PLD administered to each mouse treated by intraductal injection. Lastly, to test for any possible effects of intraductal PLD injection on neighboring mammary glands, glands contralateral to those administered PLD intraductal (n = 196 glands in 40 mice) were injected with PBS (n = 107 glands in 22 mice) or were left untreated (n = 89 glands in 18 mice). Tumor development in these glands was then compared with that in mammary glands administered PBS intraductally (n = 110 glands in 22 mice) and contralateral glands that received no treatment (n = 110 glands in 22 mice). Mice that received PLD i.v., PBS intraductally, or no treatment had a significantly greater risk (2.66-6.80 times) of developing mammary tumors than mice that received PLD intraductally (Fig. 1*E*; Table 4). Over the 16-week follow-up period, mice treated by intraductal administration of PLD developed significantly fewer tumors relative to those treated by

Table 4. Intraductal administration of PLD reduces the risk of breast tumor development in neu-N mice

Treatment comparison	No. tumors/glands treated	Hazard ratio (95% confidence interval)	*P*
PLD i.v. vs PLD intraductal	32/170 vs 13/196	2.66 (1.23-5.72)	0.013
PLD i.v. 5× vs PLD intraductal	20/120 vs 13/196	2.72 (1.22-6.03)	0.014
PBS intraductal vs PLD intraductal	69/218 vs 13/196	6.80 (3.22-14.4)	<0.0001
No treatment vs PLD intraductal	70/200 vs 13/196	6.40 (3.03-13.5)	<0.0001
PBS intraductal vs PLD i.v.	69/218 vs 32/170	2.56 (1.30-5.00)	0.007
No treatment vs PLD i.v.	70/200 vs 32/170	2.44 (1.52-3.85)	<0.0001
PBS intraductal vs PLD i.v. 5×	69/218 vs 20/120	2.50 (1.22-5.26)	0.013
No treatment vs PLD i.v. 5×	70/200 vs 20/120	2.38 (1.41-4.00)	0.001
PLD i.v. vs PLD i.v. 5×	32/170 vs 20/120	0.98 (0.60-1.60)	0.928
PBS intraductal vs no treatment	69/218 vs 70/200	1.06 (0.63-1.80)	0.820

NOTE: Six-month-old female neu-N mice were administered intraductal or i.v. injections of PLD (40 μg), PBS, or were left untreated. Intraductal injections of PLD or PBS were administered in one mammary gland per week beginning with the cervical gland in week 0 and ending with the inguinal gland in week 4. I.v. injections were administered either once or once per week for 5 weeks. Tumor development was assessed over a period of 4 months. *P* values and 95% confidence intervals of hazard ratio among listed treatment comparisons were determined by Cox proportional hazards model, where the correlation due to measurements collected on glands of the same mouse was accounted for.

i.v. administration, with 13 tumors developing in 196 treated glands compared with 32 tumors in 170 glands, respectively, which indicated that mice treated by i.v. administration had 2.7 times greater risk of developing tumors than those treated by intraductal administration of PLD (hazard ratio, 2.66; $P = 0.013$; Table 4). Notably, intraductal treatment in mice completely inhibited tumor formation for 12 weeks, after which palpable tumors were detected. In sharp contrast, i.v. treated mice developed tumors at relatively early time points after treatment. More rapid development of tumors in the contralateral untreated or PBS-treated glands limited the observation period to 16 weeks, because large tumor sizes in this group forced termination of the experiment. Although i.v. administration of PLD was not as effective as intraductal administration, it significantly reduced the risk of tumor development relative to animals that received PBS intraductal (hazard ratio, 2.56; $P = 0.007$) or no treatment (hazard ratio, 2.44; $P < 0.0001$). However, increasing the scheduling of PLD to five i.v. injections over 5 weeks did not result in a significant improvement in tumor prevention relative to mice that received a single i.v. injection of PLD (Table 4). Interestingly, contralateral PLD injection had no significant effect on tumor development in PBS-treated or untreated glands (data not shown). This observation also suggested that intraductal administration of PLD limits systemic drug exposure.

Repeated intraductal administration prolongs protection offered by PLD. To test the concept that the period of protection against tumor appearance offered by PLD could be extended by repeated therapy, 6-month-old post-breeder neu-N mice without palpable tumors received 40 μg of PLD intraductally twice per gland; all 10 glands were injected ($n = 10$ mice). Intraductal PLD injections were done bilaterally in two glands per week starting with the inguinal glands on week 0 and ending with the cervical glands on week 4. This treatment was then repeated, again beginning with the inguinal glands on week 6 and ending with the cervical glands on week 10. As a control treatment for a total of 10 intraductal injections, 6-month-old post-breeder neu-N mice that did not have palpable tumors received weekly i.v. injections of 40 μg of PLD from week 0 to 4, which were repeated from weeks 6 to 10. A total of 91 glands in 10 mice were successfully administered two intraductal injections of PLD; nine glands not successfully injected were excluded from subsequent analysis. Mice treated with PLD intraductal had no palpable tumors 20 weeks after the initial intraductal treatment, whereas all 10 mice treated with PLD i.v. developed tumors by 20 weeks (Fig. 1*F*). Glands treated with PLD intraductally had significantly better tumor-free survival than those treated with PLD i.v. ($P < 0.0001$). None of 91 glands in mice treated with PLD intraductally were observed to have tumors by 20 weeks, whereas 29 of 100 glands in mice treated with PLD i.v. developed tumors by this time (nine mice were euthanized with large tumors by 18 weeks). Interestingly, three of nine glands that did not receive two intraductal PLD injections developed tumors by 18 weeks. These results show that repeated intraductal PLD injections provided significant protection against mammary tumor growth even in the highly oncogenic HER-2/*neu* transgenic mice.

Histopathology of PLD-treated mammary glands. Mice were administered PLD (40 μg) weekly for 5 weeks by either intraductal or i.v. injection. Intraductal injections were administered beginning with the cervical gland in week 0 and ending with the inguinal gland in week 4. Histopathologic examination of H&E-stained, paraffin-embedded sections of mammary glands revealed moderate levels of inflammation in glands that received PLD by intraductal injection, as evidenced by the presence of eosinophils and neutrophils with minor lymphocytic infiltration (data not shown). Inflammation was most apparent in the glands removed 1 week after treatment with PLD and resolved over time, becoming negligible in the glands by 4 weeks. No differences were observed in whole mounts of mammary glands 4 weeks after treatment by intraductal injection relative to glands treated by i.v. injection, or in untreated controls. Six months following three intraductal injections of PLD (100 μg/duct, total of 400 μg/rat), the liver, kidney, heart, spleen, lung, and mammary glands of five PLD-treated and five untreated rats were removed. No significant histopathologic changes were observed in sections of heart, liver, lung, or kidney. Thus, intraductal administration of PLD led to a transient local inflammatory reaction in the mammary gland, which resolved within 4 weeks, and resulted in no significant long-term histopathologic changes.

Discussion

In this article, we have presented, for the first time, strong evidence that intraductal administration of antitumor agents is highly effective in both cancer prevention and therapy. Using two well-characterized models of mammary cancer (the chemically induced rat mammary tumor model and the autochthonous neu/N mouse transgenic model), we have shown the effectiveness of two anticancer drugs (4-OHT and PLD) administered intraductally. Tumor incidence and responses were significantly better than the traditional i.v. route. The apparent lack of toxicity suggests that this approach could have potential for translation to the clinic.

The greater effectiveness of drugs delivered by intraductal route compared with the systemic route was predictable. Higher local concentration of the drugs may result in more potent killing of the dividing population of epithelial cells in the mammary gland. Because proliferative lesions are precursors of cancer, localized drug-mediated clearing of the intraductal preneoplasias could prevent the development of frank malignancy. We have shown that intraductal administration of the active metabolite of tamoxifen, 4-OHT, to rats was as effective in the prevention of mammary tumors as the s.c. administration of tamoxifen, showing that agents delivered by the intraductal route can reach the preneoplastic cell population. Moreover, intraductal administration increased the efficacy of PLD in the prevention and treatment of mammary tumors in the neu-N transgenic mice compared with i.v. administration. Thus, not only does the drug reach the preneoplastic cells, but apparently, a higher concentration of the drug reaches the tumor site by intraductal compared with the i.v. route of administration.

Do these preclinical studies have potential for translation to the prevention and/or treatment of preneoplastic disease in humans? The widespread use of mammography as a screening tool has played a major role in the early detection of breast cancer and a reduction in the mortality and morbidity associated with the disease (18). One notable change attributable to routine mammography is the increase in the detection of DCIS. Just 1% before 1985, DCIS now constitutes 20% to 40% of new cases of nonpalpable breast cancer diagnosed with mammography, which in 2005 will translate to >58,500 new cases. The current treatment for DCIS includes surgical excision, with or without radiation therapy or a mastectomy. Large extensive DCIS, in particular, is difficult to manage. Although readily detected by mammography,

accurate resection of these lesions is difficult, and mastectomy is often necessary. These treatments alter breast appearance, potentially affecting body image and quality of life. Additional treatment with tamoxifen may be recommended, with frequent bothersome side effects and few but potentially life-threatening risks.

In addition to the treatment of breast tumors, intraductal injection of anticancer agents is also likely to find application in the prevention of mammary tumor development. Recently, the number of women known to be at high risk of developing breast cancer, including those having a strong family history of the disease or hereditary BRCA1 or BRCA2 mutations, has been increasing as genetic testing prevails and epidemiologic studies progress rapidly. Nevertheless, as yet, there is no effective method for preventing breast cancer development other than by prophylactic mastectomy or endocrine therapy with accompanying physical and psychologic side effects and without complete protection against breast cancer development. Few attractive options seem to be currently available for breast cancer prevention or for treatment of premalignant disease.

Before translation of this approach to the clinic, a number of additional factors, besides the inherent differences in tumor response between rodents and humans, need to be considered. First among these is the difference in the anatomy of the human breast compared with that of the rodent. The rodent mammary gland has one large central duct ending at the nipple, whereas the anatomy of the human breast is still unclear. Several small studies have produced varying results, which can be summarized by stating that there may be as many as 15 to 27 orifices at the nipple (19). In 2004, Love and Barsky used several *in vivo* and *in vitro* approaches to determine the number, distribution, and anatomic properties of the ductal system of the human breast (i.e., from the nipple orifices to the terminal duct lobular units; ref. 20). They showed that >90% of all nipples examined contained five to nine ductal orifices. These were generally arranged as a central group and a peripheral group, and each nipple orifice communicated with a separate, nonanastomosing ductal system, which extended to the terminal duct lobular unit. The results of this study suggest that most of the orifices at the nipple are not part of the ductal network, leading to sebaceous glands that lie at the periphery, whereas five to nine of the ducts surface through the nipple as milk duct orifices.

The second important issue that arises is the challenge in determining which among the five to nine duct orifices drains the affected ductal canal and must be singled out for treatment. Although a small minority of patients present with spontaneous nipple discharge that localizes the duct with the abnormality, most are asymptomatic. Advances in imaging with contrast agents infused intraductally are needed to allow us to identify the affected duct. Because the goal, in addition to the treatment of the affected duct, is also prevention, it would be desirable to infuse the drug into the affected as well as all accessible ducts, thereby reducing overall risk.

Another barrier to intraductal infusion is that early preneoplastic lesions, such as intraductal hyperplasia, can potentially block the ductal structure of the breast at any location and prevent drugs infused from reaching breast parenchyma and neoplasia distal to the obstruction, thereby compromising treatment and prevention of breast cancer and DCIS. However, central necrosis quite frequently allows dye to pass through to the other side (21). Therefore, it is possible that PLD-loaded liposomes will enter the remainder of the ductal system and treat preneoplasias and incipient tumor cells at those sites. Furthermore, any liposomes that collect near the occlusion will also continue to release drug, acting upon the DCIS cells next to them, potentially alleviating obstruction on initial or repeated administration.

Taking into account practical considerations, intraductal administration of anticancer agents is possible in the clinic in an outpatient setting. At present, several centers have expertise in cannulation of the breast ducts because it is done for the purposes of galactography and for ductal lavage. Ultimately, the questions presented above can be addressed only by performing phase I trials (22).

In summary, we have presented evidence that intraductal administration of the anticancer agents, 4-OHT and PLD, is effective in both the treatment and prevention of noninvasive mammary tumors in two animal models of breast cancer. In the clinic, intraductal administration of anticancer agents will face a number of hurdles, which are likely surmountable. The ability to identify and cannulate the diseased ductal system for therapy, or the entire ductal network for the purposes of prevention, awaits a better definition of ductal anatomy and advances in ductography. This article has presented preclinical data supporting the ability to deliver anticancer agents to mammary tumors with little systemic exposure and unprecedented efficacy. In the future, this approach may allow the use of many promising anticancer agents whose clinical application is precluded due to systemic toxicity issues. Treatments that prevent recurrence without disfigurement are urgently needed for women diagnosed with DCIS. Particularly for women at high risk for breast cancer, it seems possible that periodic treatment of the ducts with agents that eradicate preneoplasias could provide protection against the disease.

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References

1. McFarlin DR, Gould MN. Rat mammary carcinogenesis induced by *in situ* expression of constitutive Raf kinase activity is prevented by tethering Raf to the plasma membrane. Carcinogenesis 2003;24:1149–53.
2. Sivaraman L, Gay J, Hilsenbeck SG, et al. Effect of selective ablation of proliferating mammary epithelial cells on MNU induced rat mammary tumorigenesis. Breast Cancer Res Treat 2002;73:75–83.
3. Okugawa H, Yamamoto D, Uemura Y, et al. Effect of perductal paclitaxel exposure on the development of MNU-induced mammary carcinoma in female S-D rats. Breast Cancer Res Treat 2005;91:29–34.
4. Thompson HJ, McGinley JN, Rothhammer K, Singh M. Rapid induction of mammary intraductal proliferations, ductal carcinoma *in situ* and carcinomas by the injection of sexually immature female rats with 1-methyl-1-nitrosourea. Carcinogenesis 1995;16:2407–11.
5. Leonard GD, Swain SM. Ductal carcinoma *in situ*, complexities and challenges. J Natl Cancer Inst 2004;96:906–20.
6. Thompson HJ, McGinley JN, Wolfe P, Singh M, Steele VE, Kelloff GJ. Temporal sequence of mammary intraductal proliferations, ductal carcinomas *in situ* and

adenocarcinomas induced by 1-methyl-1-nitrosourea in rats. Carcinogenesis 1998;19:2181–5.
7. Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system *in vitro*: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 2004;310:1062–75.
8. Osborne MP, Ruperto JF, Crowe JP, Rosen PP, Telang NT. Effect of tamoxifen on preneoplastic cell proliferation in *N*-nitroso-*N*-methylurea-induced mammary carcinogenesis. Cancer Res 1992;52:1477–80.
9. Martin G, Melito G, Rivera E, et al. Effect of tamoxifen on intraperitoneal *N*-nitroso-*N*-methylurea induced tumors. Cancer Lett 1996;100:227–34.
10. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ. Comparative study of human and rat mammary tumorigenesis. Lab Invest 1990;62:244–78.
11. Williams JC, Gusterson B, Humphreys J, et al. *N*-methyl-*N*-nitrosourea-induced rat mammary tumors. Hormone responsiveness but lack of spontaneous metastasis. J Natl Cancer Inst 1981;66:147–55.
12. Gabizon AA. Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. Cancer Invest 2001;19:424–36.
13. Lasic DD. Doxorubicin in sterically stabilized liposomes. Nature 1996;380:561–2.
14. Sparano JA, Malik U, Rajdev L, Sarta C, Hopkins U, Wolff AC. Phase I trial of pegylated liposomal doxorubicin and docetaxel in advanced breast cancer. J Clin Oncol 2001;19:3117–25.
15. Matsui W, Huff CA, Vala M, Barber J, Smith BD, Jones RJ. Anti-tumour activity of interferon-α in multiple myeloma: role of interleukin 6 and tumor cell differentiation. Br J Haematol 2003;121:251–8.
16. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992;89:10578–82.
17. Cardiff RD. Validity of mouse mammary tumour models for human breast cancer: comparative pathology. Microsc Res Tech 2001;52:224–30.
18. Berry DA, Cronin KA, Plevritis SK, et al. Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med 2005;353:1784–92.
19. Going JJ, Moffat DF. Escaping from Flatland: clinical and biological aspects of human mammary duct anatomy in three dimensions. J Pathol 2004;203:538–44.
20. Love SM, Barsky SH. Anatomy of the nipple and breast ducts revisited. Cancer 2004;101:1947–57.
21. Love SM, Barsky SH. Breast-duct endoscopy to study stages of cancerous breast disease. Lancet 1996;348:997–9.
22. King BL, Love SM, Rochman S, Kim JA. The Fourth International Symposium on the Intraductal Approach to Breast Cancer, Santa Barbara, California, 10-13 March 2005. Breast Cancer Res 2005;7:198–204.