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Award Number: DAMD17-99-1-9242

TITLE: Prevention of Breast Cancer by Targeted Disruption of
Breast Epithelial Cells

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REPORT DATE: September 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2002	3. REPORT TYPE AND DATES COVERED Final (1 Sep 99 - 31 Aug 02)
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4. TITLE AND SUBTITLE Prevention of Breast Cancer by Targeted Disruption of Breast Epithelial Cells	5. FUNDING NUMBERS DAMD17-99-1-9242
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6. AUTHOR(S) Saraswati V. Sukumar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University School of Medicine Baltimore, Maryland 21205-2196 E-MAIL: sukumsa@jhmi.edu	8. PERFORMING ORGANIZATION REPORT NUMBER
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSORING / MONITORING AGENCY REPORT NUMBER
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20021230 128

11. SUPPLEMENTARY NOTES report contains color
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12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited	12b. DISTRIBUTION CODE
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13. ABSTRACT (Maximum 200 Words)
We proposed to test the validity of the hypothesis that introduction of recombinant toxins into the confines of the mammary ductal tree through the teat will kill breast epithelial cells. The toxins TGF- α and Heregulin-linked Pseudomonas exotoxin would be tested in the rat MNU-induced mammary tumor model. In the first year, we injected varying amounts of the TGF- α /PE and the Heregulin/PE toxin in rats by the intraductal route. While the toxin was extremely potent in human normal and breast cancer cells in culture, it was ineffective in killing the rat ductal cells. Although highly conserved, the human ligands do not appear to bind to the rodent receptors. We needed to design new toxins to target rat cells. We have completed the construction of a chimeric toxin consisting of the protein transduction domain of the HIV TAT gene to target and enter the cells, and the VPR gene of HIV to cause apoptosis. Expression of this protein in bacteria, and its purification is in progress. We have also demonstrated the efficacy of the intraductal route to cancer prevention and therapy using the NMU mammary tumor model

14. SUBJECT TERMS breast cancer, HIV, breast epithelial cells	15. NUMBER OF PAGES 26
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited
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BACKGROUND: Reliable intermediate biological markers for breast cancer risk, that can be easily detected in both pre- and post-menopausal women, do not exist at the present time. For more than 20 years, the ability to access breast ductal fluid through the nipple has prompted initiatives to develop a PAP-like test for breast cancer. Yields were variable, not every woman yielded fluid, and there was no assurance of obtaining samples from the entire length of the ducts. In this proposal, we will use a 1) a facile ductal lavage (DL) technique using cannulating catheters which flushes each duct to yield thousands of ductal cells. 2) a panel of markers consisting of three genes, Cyclin D2, Twist and retinoic acid receptor β 2 (RAR β 2), which are aberrantly hypermethylated in breast cancer cells. We will standardize the techniques using fluid from cancer patients, and then evaluate the frequency of cells positive by MSP assays in ductal lavage obtained from women with a high risk of developing breast cancer, such as patients with lobular carcinoma, patients with cancer in one breast, and those with mammographically suspicious lesions. Thus, we aim to develop a PAP test for the breast. Women with a strong family history of breast cancer may have up to an 80% risk of developing breast cancer over their lifetime. Given the rising risk, and the increasingly identifiable high-risk group, the time has come to give serious consideration to the options available to prevent breast cancer. The matter has acquired a sense of urgency in the last three years because of two seminal discoveries in the genetics of hereditary breast cancer. Individuals with a family history of breast cancer (comprising 5-10% total breast cancer cases) often carry a mutation in the breast cancer susceptibility genes BRCA1, BRCA2 or p53 and ATM are at particularly high risk of developing breast cancer at a young age. As more women test positive for mutations in BRCA1 and BRCA2, the question of how best to manage these patients becomes ever more pressing. Unless reliable and effective methods for preventing breast cancer can be devised, determining susceptibility to breast cancer may be useless and possibly even psychologically detrimental. As more breast cancer associated genes are identified, particularly among the larger population of women without a strong family history, preventive strategies with minimal side effects are clearly needed.

BODY

This proposal sought to test the radically new idea that breast cancer prevention can be achieved by selectively killing the cells that line the ducts from which the majority of malignant breast cancers arise. Multiple strategies could be applied to selectively kill breast epithelial cells. One method is to use proteins called ligand/toxin conjugates. Ligand/toxin conjugates combine their cytotoxic properties with the ability to selectively target cells carrying specific growth factor receptors. Cells that do not express the receptors remain unaffected.

We initiated experiments to determine the LD50 of the toxins when administered by the novel intraductal route in rats and the dose of toxin that will effectively ablate the mammary gland. following injection of upto 2 ug of toxin per rat, no generalized toxic effects such as weight loss or death of mammary ducts as determined by microscopic examination of sections of the mammary gland was observed. we raised the dose upto 4 ug, with no effect. neither of the two toxins was effective, suggesting that neither the egf receptor or the neu receptor was binding to the cognate human ligand linked toxin. in the face of these observations, it was clear that we needed to design a new ligand/toxin that would be lend itself to testing in rodent model systems and is then translatable to humans.

Delivery of cytotoxic proteins as a means of prevention or therapy is hampered by their size and biochemical properties. Work from Steve Dowdy's laboratory has demonstrated the efficiency of delivery of large proteins by fusing them to a 12-amino acid protein transduction domain of the HIV TAT protein. This protein transduction happens in a swift, concentration-dependent fashion is independent of receptors. Instead, it targets the lipid bilayer of the membrane of the cell. So, in theory, all mammalian cell types should be susceptible to this mode of protein

transduction. Further the same work goes on to show internalization and expression of the same TAT protein linked to - \square GAL protein in various mouse organs in vivo. The purified proteins were denatured while the injected proteins were renatured intracellularly.

Another HIV gene VPR (Viral Protein) encodes a protein which induces G2 arrest and apoptosis in a variety of cell types by a direct effect on the mitochondrial permeability transition pore. Amino acids 53 to 96 of VPR encompass a basic domain that is sufficient and responsible for this apoptotic effect.

We have tried many approaches for synthesizing and purifying sufficient quantities of the TAT-VPR protein to use in animal experiments, but failed so far.

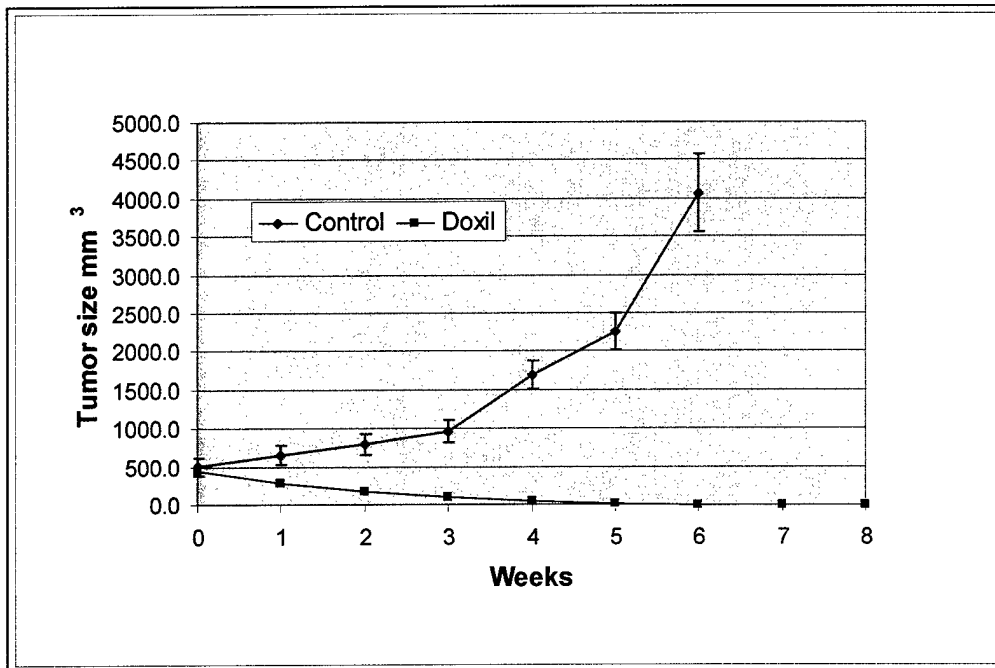
At the same time, to test the validity of the intraductal approach to prevention and therapy of breast cancer, we also initiated experiments to use simpler systems to test the efficacy of the intraductal approach to prevention and therapy using well known drugs, such as tamoxifen and doxorubicin.

We have used the well-known drug tamoxifen which has been found in clinical studies to have tumor preventive effects in women at high risk of developing breast cancer. However, liver toxicity remains a matter of grave concern in the use of tamoxifen on a long-term basis. Experiments were initiated to determine if tamoxifen (or 4-hydroxytamoxifen, its final derivative) given intraductally will provide protection against nitrosomethylurea-induced mammary carcinomas.

Sprague-Dawley rats were administered a single injection of 50mg/kg body weight of NMU. Treatment with 4-hydroxytamoxifen was initiated after 2 days. Rats were administered 50 μ l of 1 μ g/ μ l of the drugs at weekly intervals for 4 weeks. Animals were followed up for 12 months with weekly palpations to detect tumors. Results are shown in Table 1. the experiment was performed twice and the results are shown as cumulative numbers for the two experiments. Tamoxifen alone, and oil alone (used as a negative controls in experiment 2) had no effect, while 4-OH Tamoxifen had potent tumor preventive effects. Injection of tamoxifen s.c (positive control) was highly effective as a tumor preventive, as previously shown by others. Thus, using the metabolically active 4-OH tamoxifen given locally to the ductal cells was highly effective against development of mammary carcinomas in rats given NMU.

Treatment	n	# of ducts injected	# of tumors	p value
No treatment	24	288	62	
4 OHT i.duc	20	201	1	<0.0001
TAM i.duc	10	98	17	0.5672
Oil i.duc	6	50	7	0.4161
Tamoxifen s.c	20		0	<0.0001

In the next series of experiments, we tested whether administration of drugs via the milk duct is effective in the elimination of established tumors. For this purpose, Doxil, which is a liposomal preparation of doxorubicin was used. NMU- induced rat mammary tumors were treated when their size reached 10mm. Fifty microliters of Doxil (2mg/ml) was administered once weekly via the duct. With the exception of one tumor which did not respond to Doxil, 11/12 tumor-bearing animals responded to the drug and remained tumor-free for over a period of 4 months of follow-up. All 12 tumors that were treated with control liposome continued to grow (see Table below). Thus, the intraductal route of administration of Doxil proved very efficacious in effecting a cure. This appears to be a useful route that may prove as efficient as the systemic route but would lack the side-effects.



•Tumor size at initiation of treatment: approx.10x10x5 mm, 12 rats per group
•Once weekly injection of Doxil (2mg/ml) for two weeks.

KEY RESEARCH ACCOMPLISHMENTS

- synthesized the TAT-VPR chimeric gene using oligosynthesis. Cloned the gene into an expression plasmid.
- Showed tumor- preventive properties of 4-hydroxytamoxifen given intraductally to Nmu-treated rats
- Showed tumor-therapeutic properties of Doxil, a liposomal preparation of Doxorubicin against established breast carcinomas in the rat.

REPORTABLE OUTCOMES

Publications: In preparation.

Presentations: Breast Spore meeting at Dana Farber Cancer Center, Boston, MA, October 2001

Breast Cancer Research Meetings, - December. 10-13, 2001, San Antonio, TX -

10th SPORE Investigators' Workshop –Early detection of breast cancer cells in ductal lavage fluid

– quantitative assessment of cyclin D2, RAR- β , Twist, RASSF1A, and HIN-1 by real time methylation specific PCR (abstract). July 13-16, 2002.

Annual Meeting of Society of Gynecology and Obstetrics, April 3-5, Innsbruck, Austria
Association of Investigative Pathologists, April 20, 2002
American Radium Society Meeting – April 27-30, 2002, Las Croabas, Puerto Rico
CYTYC Health Corporation – May 23, 2002, Boxborough, MA.
Abbott Laboratories – May 10, 2002, Abbott Park, IL

CONCLUSIONS

Detailed experimentation on the in vivo effects of TGF- α -PE toxin and Heregulin/PE toxin revealed that the toxins are specific to human cells, and had no action on rat ductal cells. No toxic effects were discernible even at very high doses of 4 ug per rat. A novel toxin was designed using reagents that contain TAT domains that are internalized by all mammalian cells and the domain of VPR that causes apoptosis in cells. The chimeric protein will be injected alone and also as liposome preparations to achieve nonsurgical removal of preneoplasias as well as dividing cells in the breast.

The intraductal route of injection may find uses in both prevention and therapy of breast cancer. We have shown that 4-hydroxytamoxifen can prevent the development of mammary carcinomas and Doxil can cause regression of well-established tumors in the NMU-induced mammary carcinoma model system.

The work has significant potential in that it aims to develop a method of preventing breast cancer by ablating the breast epithelium that is the site of origin of breast carcinomas. The right agent to achieve this goal has not been formulated yet. Effort sare underway to design and use an agent that will be non-toxic to other cells but will kill breast epithelium.

REFERENCES

None

APPENDICES

Summary of the work in a presentation format.

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DAMD17-99-1-9242

APPENDIX

Current Options in Breast Cancer Prevention

- Surveillance
 - self-examination
 - mammography
- Hormonal Regulation
 - Tamoxifen or Raloxifene
 - Retinoids
 - Gonadotropins
- Dietary
 - flax seed oils
 - genistein (soy beans)
 - limonene (citrus)
 - ω -3 fatty acids (fish oil)
- Prophylactic Mastectomy



More than 95% of breast cancers arise in the epithelial cells lining the breast ducts; however, these cells comprise only about 5-10% of total breast cells.

Our Hypothesis

- Ablation of mammary epithelium should prevent tumor formation.
- Ductal openings in the nipple provide easy access to the epithelial network. Therefore, cytotoxic agents delivered by intraductal injection through the teat should confine their effects to the mammary epithelial cells alone.

Primary Candidates for Treatment

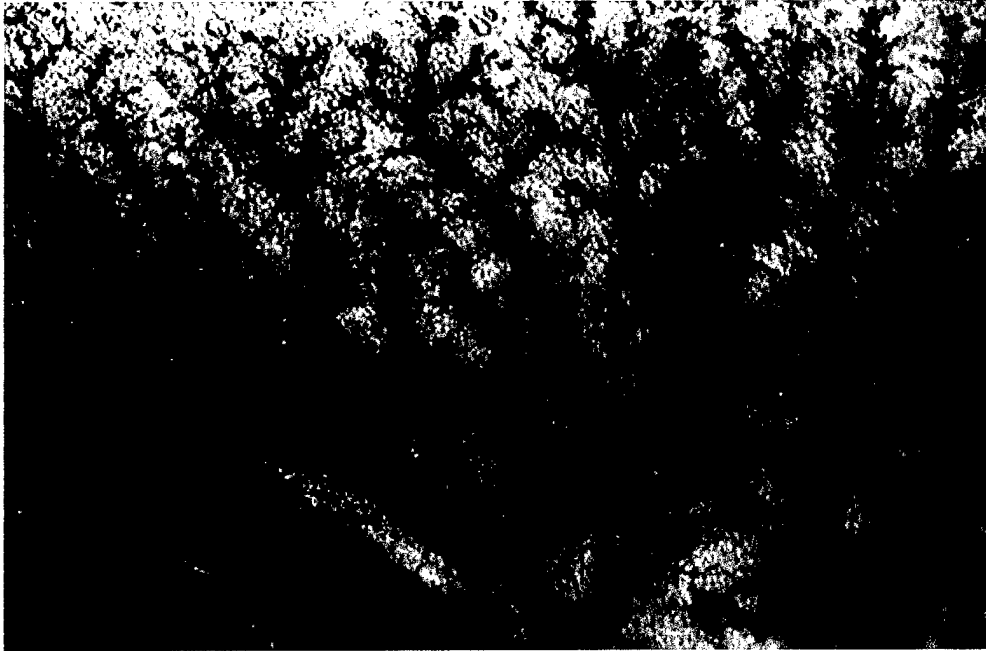
- Women who have inherited mutations in the BRCA1 or BRCA2 genes and those with a family history of breast cancer.
- Women diagnosed with lobular carcinoma *in situ*.
- Women who are considered candidates for prophylactic mastectomy.

Questions...

- Can the entire ductal tree be accessed by cannulating the major ducts?
- Can the cytotoxicity of the epithelial cells be maximized while minimizing the local and systemic toxicity?
- Does this procedure provide protection from breast cancer?

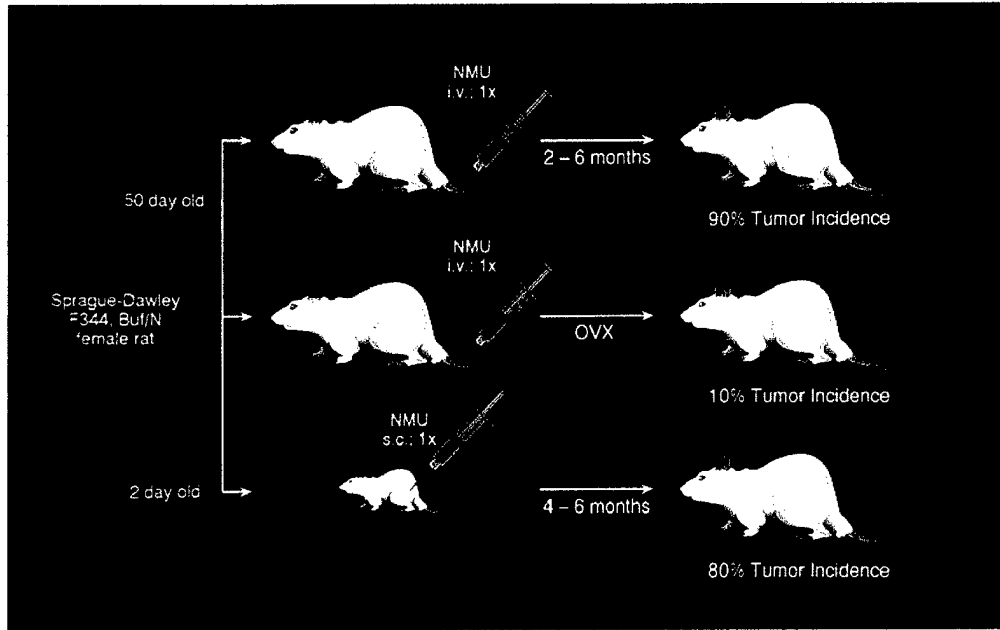
The mammary ducts are accessible from the teat.....





- Does ablation of ductal epithelium prevent mammary tumorigenesis?

The nitrosomethylurea-induced rat mammary tumor model system



Multiple stages of breast cancer are represented in the lesions in the mammary gland of NMU-treated rats within 2-3 months- ADH, DCIS, and intraductal carcinomas.

Induction of intraductal proliferations, ductal carcinoma *in situ* and carcinomas in abdominal-inguinal mammary glands

Histology classification	Number of lesions	Percent of total lesions
IDP	37	26.6
DCIS	16	11.5
AC-predominantly DCIS	47	33.8
AC-no DCIS component	39	28.1
Total	139	100.0

These data are based on the lesions excised from the abdominal-inguinal mammary glands of a group of 30 rats. Singh et al, Carcinogenesis, 1992

Epithelial cell-ablation strategies....

1. Adenoviral-HTK delivery and treatment with GCV
2. Vaccinia virus carrying lac-z
3. Pseudomonas exotoxin linked to TGF-alpha to target EGF receptors on epithelial cells.

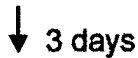
Typical Adenovirus-HTK Protocol

Inject major ducts with Adeno-HTK (Ad-5 RSV promoter, Ad-5 CMV promoter)

Estrogen administration (5 mg/kg i.m.)

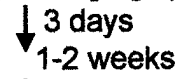


Administer Ganciclovir (150 mg/kg i.p. twice daily for 3 days)



Estrogen administration (5 mg/kg i.m.)

Administer Ganciclovir (100 mg/kg i.p. once daily for 3 days)



Administer NMU (50 m/kg i.v. or i.p.)



Protection by Adenoviral-HTK/GCV Treatment

Treatment	Total Rats	Rats with Tumor	Percent
Untreated Control	6	5	83
E ₂ , Adeno-HTK	6	5	83
E ₂ , Adeno-HTK, GCV	8	1	13*

*p<0.005

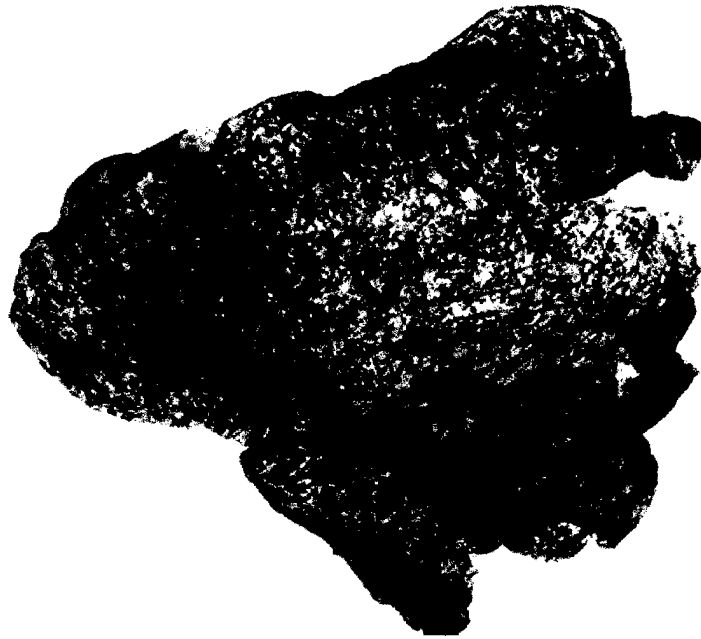
Innovation

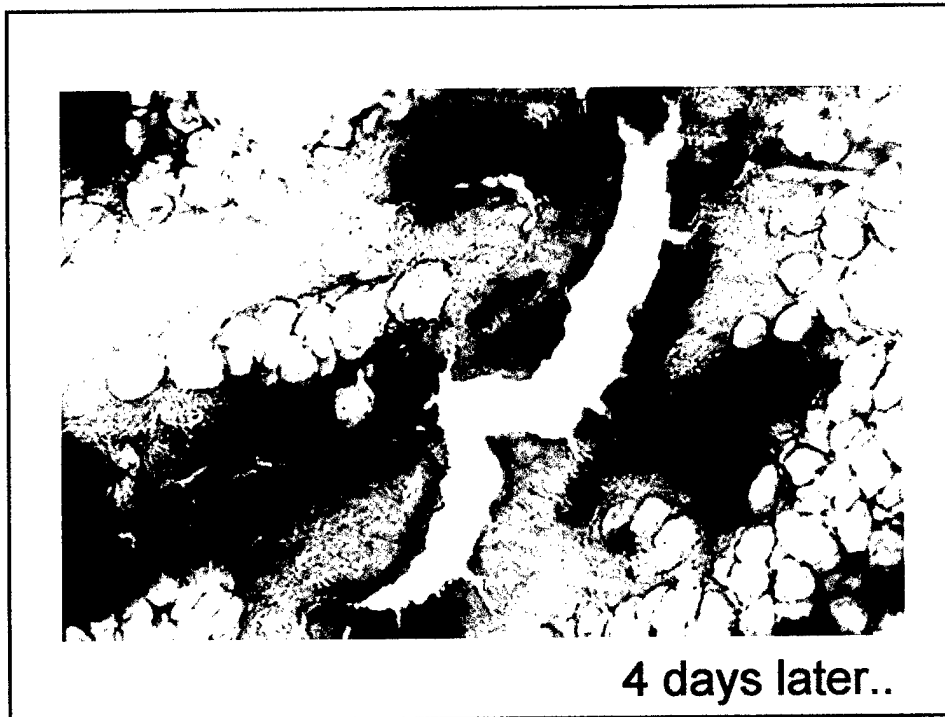
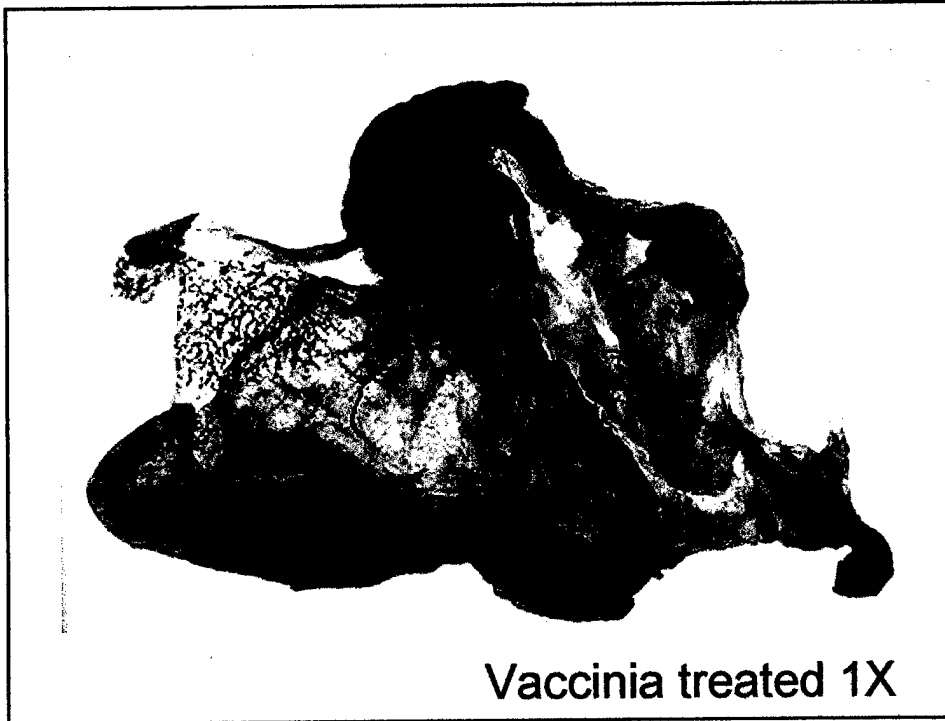
- **A strategy to combine prevention and therapy**
- **Inject retroviral-HTK into young pre-pubertal mammary gland to achieve sustained expression throughout life in a large population of cells.**
- **Inject ganciclovir anytime during the lifetime of the animal to initiate selective mammary epithelial cell death.**

Problems-

1. **Inefficient rate of gene transfer**
2. **Reducing number but inefficient killing of the mammary gland cells**
3. **Re-growth, in time, of the remnant mammary gland elements.**
4. **Susceptible to more doses of carcinogen.**

Effect of intraductal delivery of vaccinia virus-Control gland

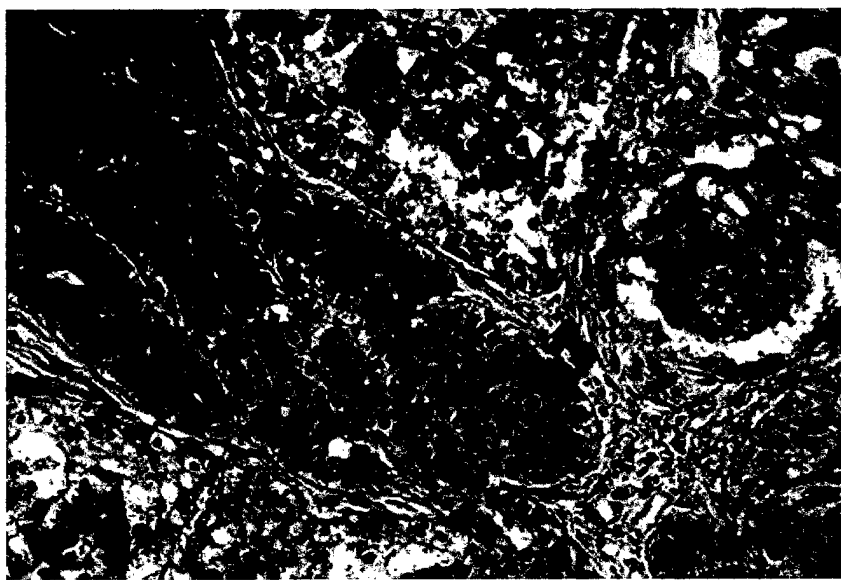




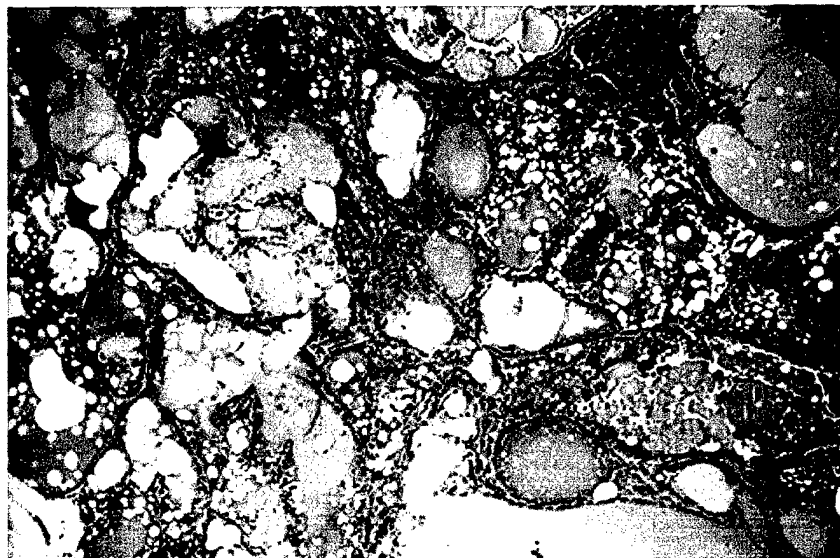


10 days later...

**NMU-induced mammary tumors-
intraductal carcinomas**



Tumor cell lysis and necrosis in vaccinia-treated mammary tumors



Injection of live vaccinia i.d as a prevention strategy- results

- 1. Very efficient ablation of epithelial elements within very a short time.**
- 2. Effectively reduces tumor incidence in treated rats (0/6) versus untreated (7/12) rats.**
- 3. Causes severe inflammatory reaction and mammary elements grow back within 1-2 months.**

The TGF- α /PE toxin

Highly effective in killing cultured human and rat carcinoma cell lines and normal epithelial cells in culture. But the PE-toxin was totally ineffective and non-toxic to both mice and rat epithelium and to the whole animal.

Possible reason: No EGF receptors on luminal surface? EGF receptors in rodents will not bind human TGF- α ?

Re-think strategy....may not need total epithelial cell ablation but removal of pre-neoplastic lesions from the breast- a model for prevention alongside therapy

Proof of Principle:

- Intraductal treatment with known drug 4-OH tamoxifen, before and after tumor formation.
- Intraductal treatment of established tumors using Doxil.

Intraductal 4-hydroxytamoxifen prevents mammary carcinogenesis

Treatment	n	# of ducts injected	# of tumors	p value
No treatment	24	288	62	
4 OHT i.duc	20	201	1	<0.0001
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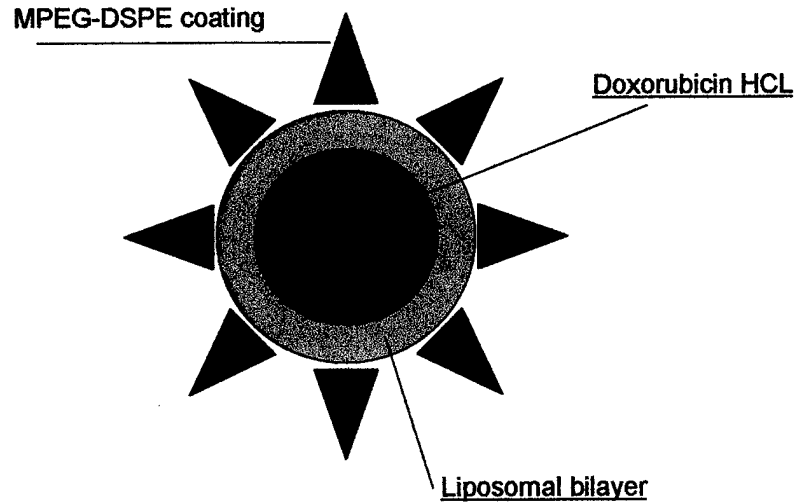
Dose of 4-OHT and Tamoxifen 1ug/ul, 50 ul per injection, weekly for 4 months

The intraductal approach to targeted and localized delivery of therapy

- Surgimimetic.
- Local administration of therapy, reducing undesirable side-effects.
- Injury and discomfort kept to a minimum.
- Repetitive delivery for maximum efficacy.

Test case: Doxil

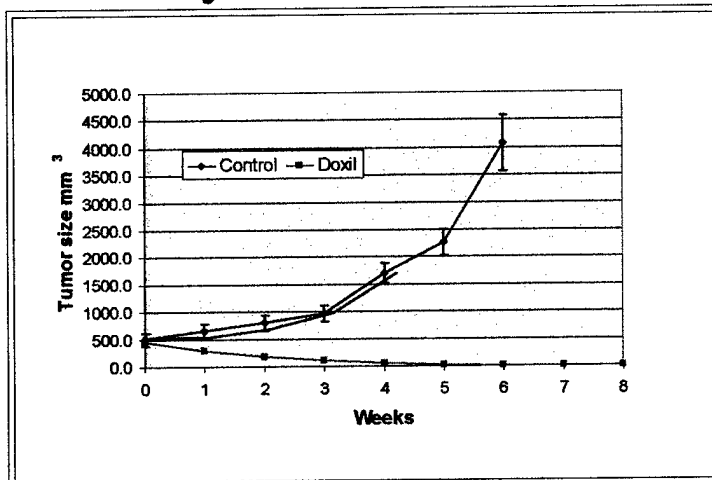
STEALTH LIPOSOME



DOXIL

- Doxorubicin as a liposomal preparation is not a vesicant (causes burn-like lesions)
- Pegylation (Surface bound methoxy polyethylene)
- Helps avoid immune system and stays active longer
- Liposomal formulation helps prevent toxicity.

Treatment of established mammary tumors with Doxil i.d



- Tumor size at initiation of treatment: approx. 10x10x5 mm, 12 rats per group
- Once weekly injection of Doxil (2mg/ml) for two weeks.

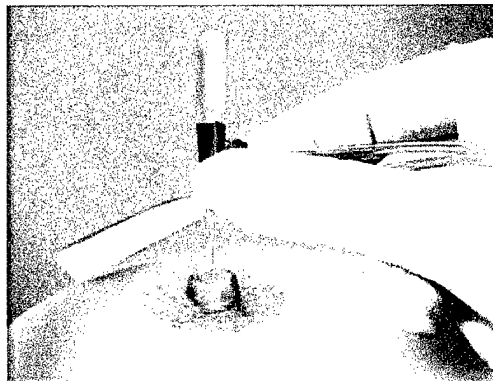
Mechanism of action- Doxil

- Active ingredient in Doxil is Doxorubicin HCl.
- Small size (100nm) and long half life enable penetration to altered tumor microenvironment and accumulation inside tumors.
- Concentration of drug 20X higher in tumors when given i.v.
- Mode of release of doxorubicin from liposomal preparation not understood, but tumor pH change thought to be partially responsible.

Translational potential?

- Does this approach have translational potential?
- What platforms can it be tested in?
- Chemotherapy prior to surgery... compare systemic versus same dose intraductal therapy, followed by surgery. Endpoint: faster or greater change in tumor size?
- Combine targeted toxin-based therapy i.d with conventional systemic chemotherapy
- This concept has gained validity because...

A technique to access ducts- duct catheters



- Microcatheter introduced into ductal opening
- For ductal lavage, 10-20 mL of saline slowly introduced through the catheter in 2 mL increments
- This approach can be adapted to administering drugs of choice

Prevention strategies....

Will agents such as Doxil administered at regular intervals prevent growth of preneoplasias and invasive cancers in high risk women? The route will minimize toxicity and maximize targeted delivery and effectiveness.

Will technique be useful for treating all women post-childbearing to prevent breast cancer?

Conclusions and future plans...

1. Prevention and therapy of mammary tumors is feasible due to local and field effects of Doxil and 4-OHT
2. Delivering molecules into all the cells in the duct - TAT, Trojan peptides etc. linked to molecules that would induce apoptosis.
3. Liposomal formulations that would release the active drug in place over a period of weeks.