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13. ABSTRACT (Maximum 200 Words)

This project will evaluate paclitaxel chemotherapy delivery from a gel polymer system placed into a wound bed following conservative (marginal) surgical removal of human breast cancers grown in nude mice. This delivery method is proposed to control local tumor disease as well as assist in control of systemic metastasis. We have established 5 human breast cancer cell lines within our laboratory. We have elected purchase and implement a unique (luciferase) imaging system which allows in vivo imaging of tumor growth and metastasis (and subsequently decrease animal use). Tumor cell lines have been transfected with the luciferase gene. In vitro testing of cell lines has established *paclitaxel* sensitivity and shown a synergistic effect of delivering *paclitaxel* by the poloxamer polymer for the chemotherapy resistant cell line, MCF-7-ADR. We have begun the simultaneous evaluation of local and systemic toxicity, local, regional and systemic distribution and local and systemic efficacy of locally delivered paclitaxel chemotherapy following tumor removal using the MCF-7-ADR cell line in nude mice. Intracavitary administration of taxol in poloxamer has been well tolerated and resulted in complete control of local tumor regrowth and metastasis following marginal tumor removal. This compared to only marginal tumor control using systemically delivered parent paclitaxel.

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Annual Report for Award Number DAMD17-02-1-0347, 4/27/2004

William S. Dernell DVM, MS

Introduction: This proposal will evaluate paclitaxel (taxol) chemotherapy delivery from a gel polymer system (poloxamer 407, Pluronic F127, BASF) placed into a wound bed following conservative surgical removal of human breast cancers grown in nude mice. This novel delivery method is proposed to control local tumor disease as well as assist in control of metastasis and may offer a cost-effective alternative to adjuvant radiation therapy.

Body: Task (objective) 1 (proposed to be completed in year 1): *To evaluate the efficacy of polymer delivered paclitaxel (taxol) chemotherapy against human breast tumor cell lines.* As per task 1, we have established 5 human breast cancer cell lines within our laboratory;MCF-7, MCF-7 AL, MDA-MB-435, MDA-MB-231 and MX-1. Sensitivity (LC5) to taxol as well as poloxamer 407 - taxol mixture (polotax) was determined in vitro in four human breast tumor cell lines: MCF-7, MCF-7/Adr, MDA-MB-231, and MDA-MB-435. MTS-assays (Celltiter 96 Aqueous one solution cell proliferation assay - Promega) in 96 well plates were used, each well received 100 ul of cell culture medium and 5,000 cells of the respective cell lines (**Figure 1**).

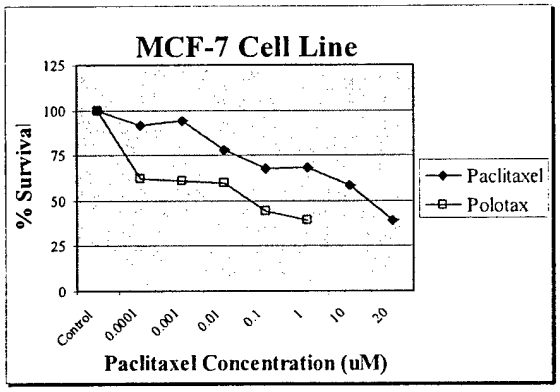


Figure 1 A

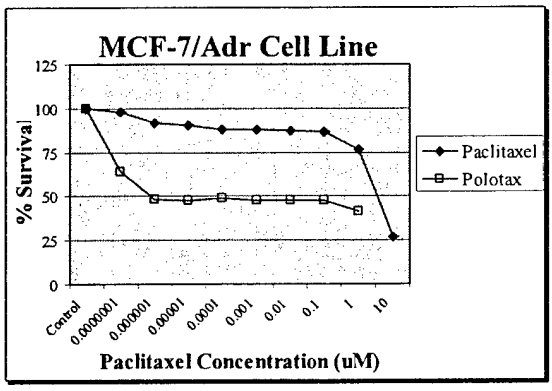


Figure 1B

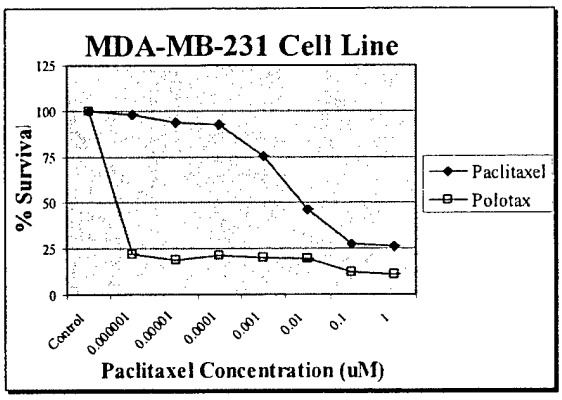


Figure 1C

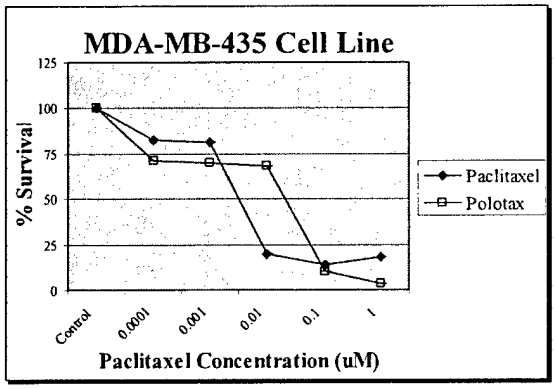


Figure 1D

Figure 1.
Cell survival curves for 4 human breast tumor cell lines exposed to various concentrations of taxol and poloxamer-taxol (polotax) combination.

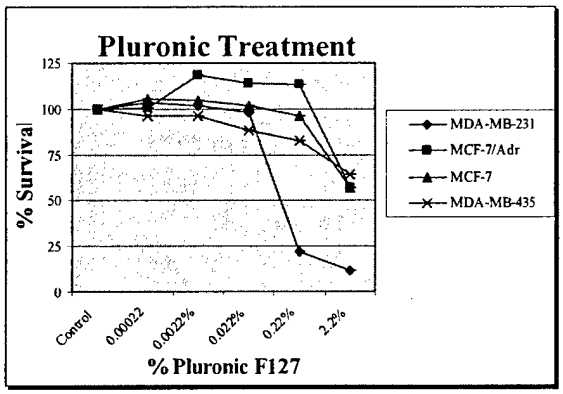


Figure 2

Figure 2.
Cell survival curves for 4 human breast cancer cell lines exposed to various concentrations of poloxamer 407 (Pluronic F127)

Results of In Vitro Studies.

a) Taxol treatment alone produced LC50 values of 20μM, 10μM, 0.01μM, and 0.01μM for MCF-7, MCF-7/Adr, MDA-MB-231, and MDA-MB-435 respectively.
b) Treatment with polotax resulted in LC50 values of 0.1μM, 0.000001μM, 0.000001μM, and 0.1μM, respectively.
   - Three of the four cell lines studied clearly demonstrate that paclitaxel delivered via Pluronic F-127 is more effective than the drug delivered alone.
   - We hypothesize that the polotax data is the result of a synergistic relationship between the paclitaxel chemotherapy and the polymer delivery system.
   - The pluronic F-127 itself had inherent cytotoxic effects, supporting previous investigator findings. This polymer also appears to sensitize cells to chemotherapeutics **(Figure 2)**.
   - Pluronic F-127 appears to sensitize the multidrug resistant (MDR) MCF7-Adr cell line to paclitaxel relative to the parental MCF7 cell line.
   - Potential mechanisms of MDR cell sensitization by polymers could include:
     - Alteration of membrane viscosity
     - Abolish sequestration of drug in cytoplasmic vesicles
     - Depletion of ATP

To test the hypothesis of ATP depletion, luciferase transfected MCF-7-Adr cells were incubated with Pluronic F127, then exposed to luciferin. The reaction of luciferine with luciferase to yield light photons is ATP dependent. If Pluronic inhibited ATP, then a decrease in liciferin reaction should occur, dependent on the amount of pluronic exposure. **Figure 3**, demonstrates this relationship.

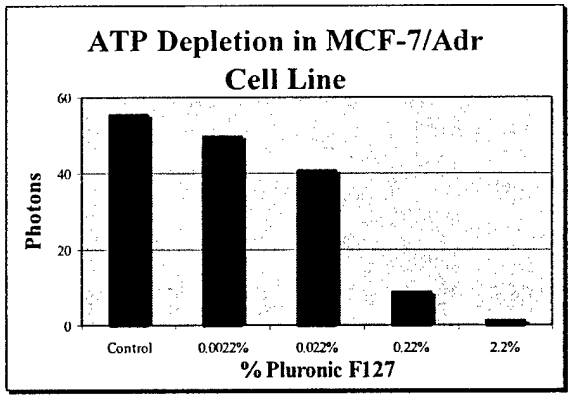


Figure 3

Figure 3.

Photon emission from luciferase transfected MCF-t-Adr cells exposed to luciferin after 24 hrs exposure to pluronic F-127 results in reduced photon emission at higher concentrations, suggesting the ATP depleting effect of pluronic F-127.

We have elected to purchase and implement a unique luciferase imaging system (not in original proposal and not paid for using grant monies from this award) which allows in vivo imaging of tumor growth and metastasis. The following paragraph describes how this system is implemented:

Tumor growth is evaluated by IVIS technology. Briefly, animals are anesthetized by i.p. injection of 40 ul of a ketamine and xylazine (4:1) solution. An aqueous solution of the substrate luciferin (the substrate for luciferase, Molecular Probes, 50mM, 126mg/kg) is administered by

intraperitoneal injection 5 min before imaging (Sweeney et al., 1999). Supine mice are then placed into a light-tight specimen chamber mounted with the charge-coupled device (CCD)-camera cooled to -120°C. A gray-scale body-surface reference image is collected first followed by acquisition of the photons transmitted from the luciferase transfected cells in the mice. Using LIVINGIMAGE software (Xenogen), overlay of the pseudocolor image represents the spatial distribution of photon counts. Signal intensity is quantified as the sum of all detected photon counts within the region of interest after subtraction of background luminescence measured at shoulder level (Vooijs et al., 2002, Hudachek et al., 2003).

Use of this system allows evaluation of disease progression (and response to treatment) without the need for animal sacrifice until the final endpoints of the study. This will significantly decrease animal use. Use of this system requires transfection of the breast tumor cell lines with the luciferase gene. We have currently transfected the MDAMB435 cell line. cell lines with the lucerferase gene prior to moving on to task 2.

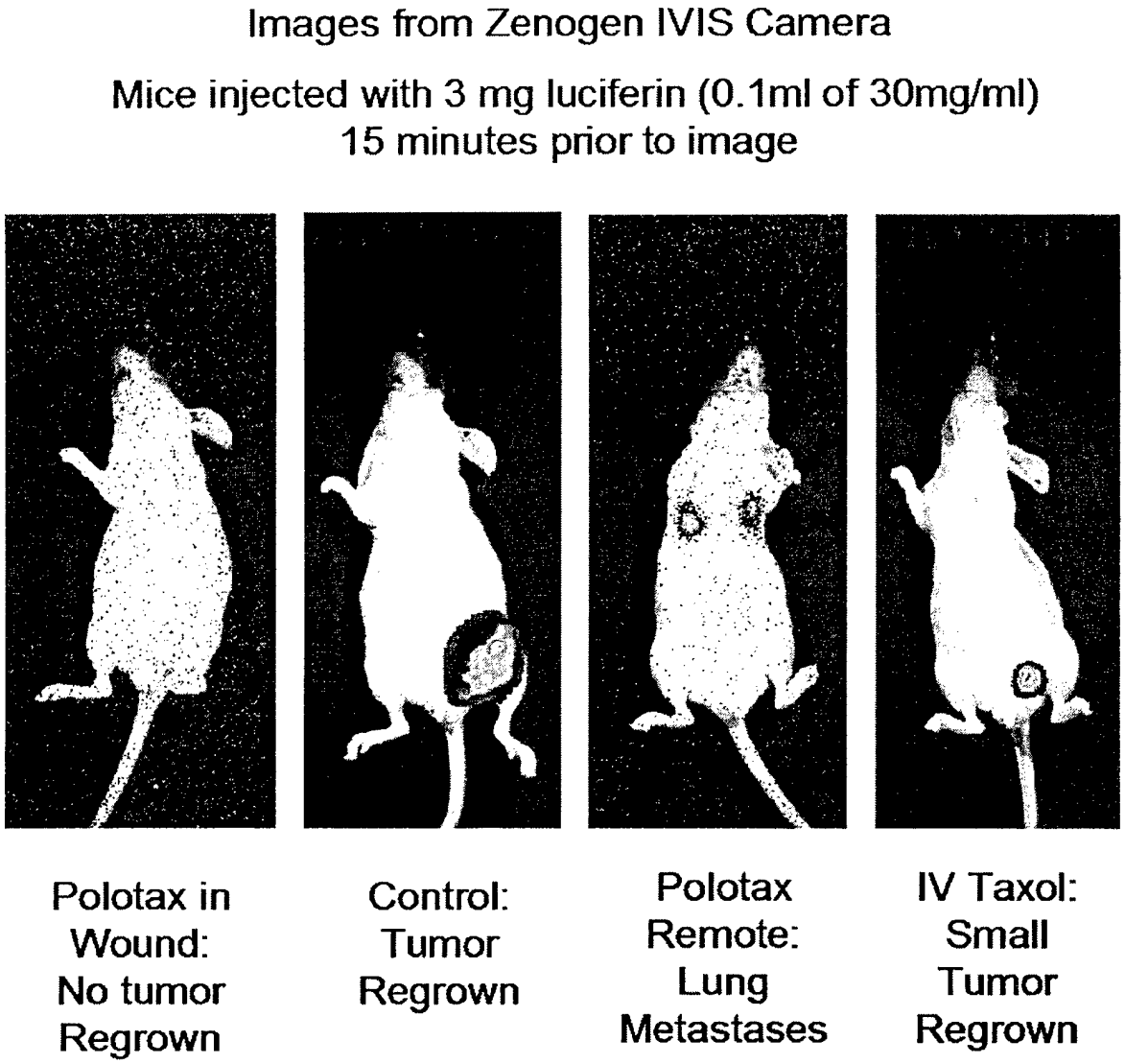
Tasks (objectives) 2-4: *To evaluate the local and systemic toxicity, the local, regional and systemic distribution and the local and systemic efficacy of locally delivered paclitaxel chemotherapy following tumor removal.* These objectives are to be evaluated simultaneously in the in vivo portion of the study. Methods and preliminary results relating to tasks 2 and 3 will be discussed following the discussion of the methods and preliminary results of task 4.

Four cell lines (MCF-7, MCF-7/Adr, MDA-MB-231, and MDA-MB-435), identified as sensitive to taxol, were stably transfected with the luciferase reporter gene. When these luciferase transfected cancer cells are exposed to the substrate luciferin, they emit light photons. With the ultrasensitive CCD-camera of our new In Vivo Imaging System (IVIS, Xenogen), we can detect these bioluminescent signals emitted from the cancer cells inside the mouse on the outside of the body of a live mouse. With this technology we are able to detect quantitatively small numbers of tumor cells and follow these over several logs of cell growth. Data is integrated with LIVINGIMAGE software (Xenogen), and tumor size signal is expressed as total photons emitted.

MCF-7/Adr is a human breast adenocarcinoma cell line, in vitro selected for adriamycin resistance. MCF-7/Adr's express a high level of P-glycoprotein pumps. In vivo tumor growth displays a highly vascularized, aggressive pattern. In vitro data on MCF-7/Adr taxol exposure (cfr supra), indicated a remarkable synergistic effect with a $10x10^6$-fold increase in sensitivity of the cell line to taxol when delivered in combination with the poloxamer 407. We subcutaneously inoculated 70 nude mice with 1 million MCF-7/Adr cells in the left inguinal mammary fat pad. Tumor growth was monitored weekly by imaging of luciferase activity with a CCD camera and LIVINGIMAGE software and by caliper measurements. Animals with immediate peritoneal metastasis (3/70) were removed from the project. Only animals with localized primary tumor growth were considered. Primary tumors were allowed to grow to 3 different size ranges: 500-800 mm^3, 400-500 mm^3 and 300-400 mm^3. At time of primary (marginal) tumor resection, animals were randomly assigned to one of 5 treatments: a) polotax (200 ul of 22% poloxamer/5.4mg/ml taxol suspension) in wound, b) 200 ul polotax remote (between 2 scapulae), c) 200 ul 22% poloxamer in wound, d) 20 mg/kg taxol IV (200 ul of 400ug taxol, 1:2 dilution of 6mg/ml Taxol in cremophor in saline (Paclitaxel, Bristol-Myers Squibb) and e) no drug control. Mice were imaged for luciferase after surgery to evaluate for tumor remnant. Subsequently, mice were imaged on a weekly schedule to evaluate tumor regrowth and tumor metastasis. By

means of this highly sensitive, non-invasive imaging method, one can image tumor dynamics over time within the same animal. In the initial proposal we planned to assess tumor regrowth at two time points (14 and 60 days), however with this new imaging modality we can assess tumor behavior on a weekly basis within the same animal, reducing the animal number for this study to half (**Figure 4**).

Figure 4.
Images of local tumor growth and pulmonary metastasis from mice implanted with MCF-7-Adr human breast tumor cells.



Results of In Vivo Experiments.

Figures 5-8 show preliminary results of local tumor regrowth and metastasis as evaluated using the luciferase imaging system. Zero of 9 and one of 9 mice treated with polotax placed within the wound (intracavitary) following marginal tumor removal has shown local regrowth by 40 and 60 days post treatment, respectively (**Figures 5 and 6**). Five of 8 and Six of 9 mice treated with intravenous taxol (parent drug) have shown tumor regrowth by 40 and 60 days post treatment, respectively (**Figures 5 and 6**). Zero of 9 and one of 9 mice treated with polotax placed within the wound (intracavitary) following marginal tumor removal have shown distant metastasis at 40 and 60 days, respectively (**Figure 7 and 8**). Two of 9 and 5 of 8 mice treated with intravenous taxol have shown metastatic failure at 40 and 60 days, respectfully (**Figure 7 and 8**). This would support a benefit to local treatment with polotax over intravenous taxol. Polotax placed at a distant sight (not within the tumor wound) showed similar control of local tumor regrowth to systemically administered taxol (**Figures 5 and 6**). These effects were seen even with large primary tumors (prior to removal) as well as tumors removed at an earlier, smaller stage. (**Figure 6**)

Figure 5.

Overall Local tumor regrowth of MCF-7-Adr human breast tumor cells following marginal removal of primary tumor in nude mice after postoperative treatments (to 40 days following treatment).

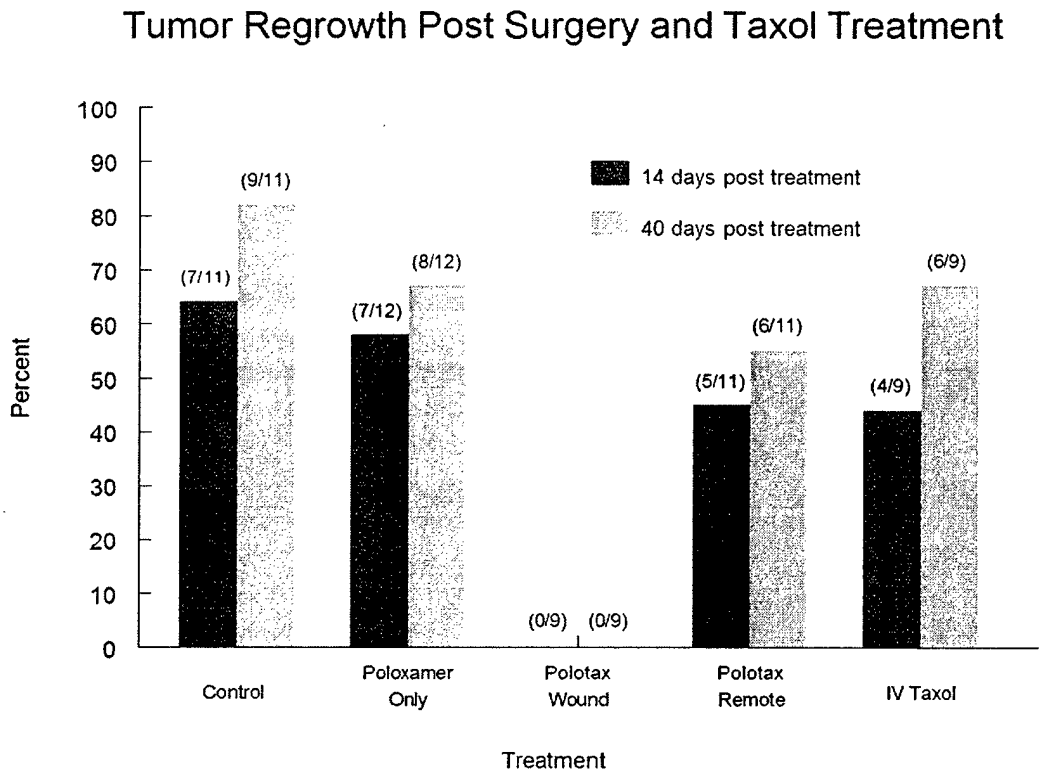


Figure 6A-C.
Local tumor regrowth of MCF-7-Adr human breast tumor cells following marginal removal of primary tumor in nude mice after postoperative treatments based on size of the tumor at the time of surgical removal (to 60 days following treatment).

Figure 6A

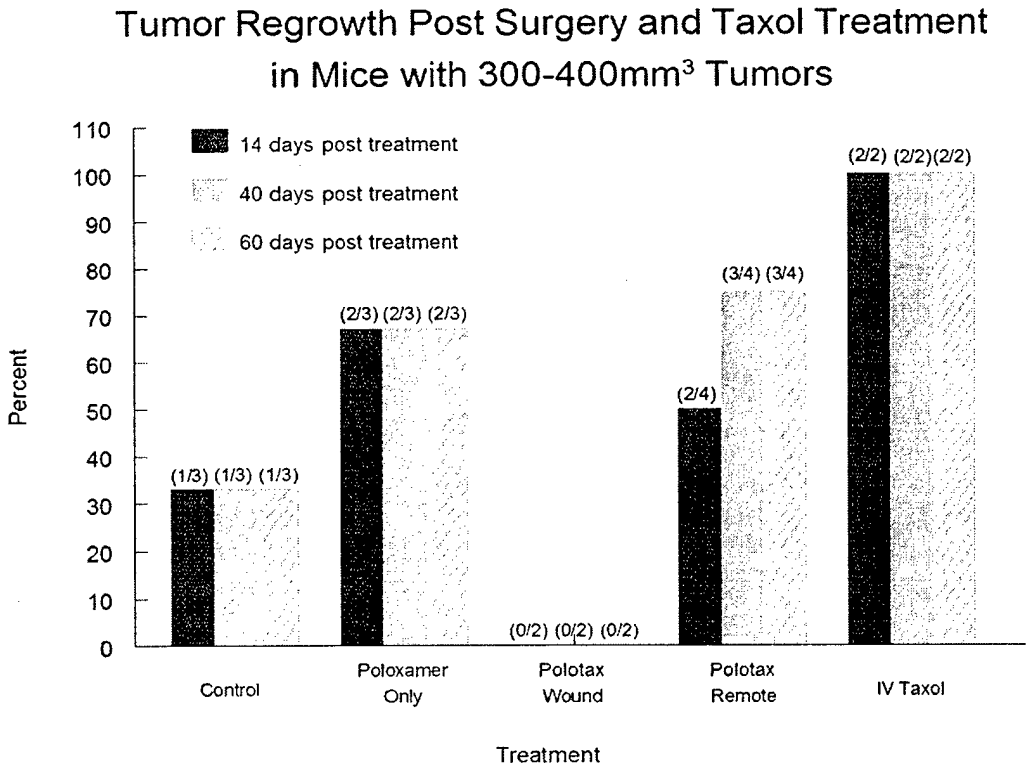


Figure 6B

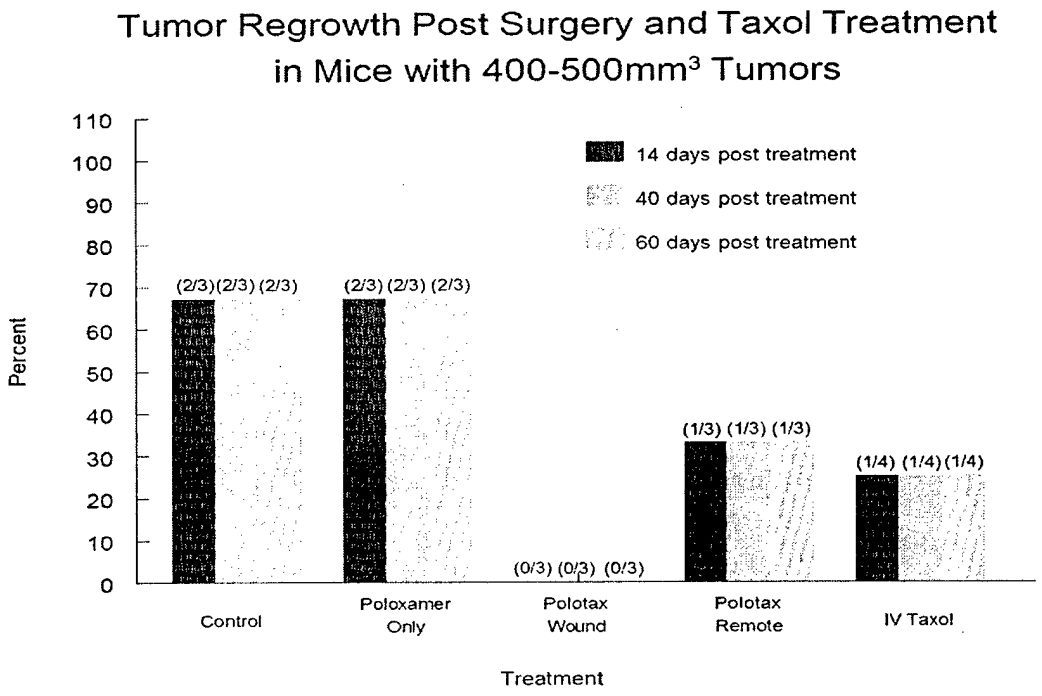


Figure 6C

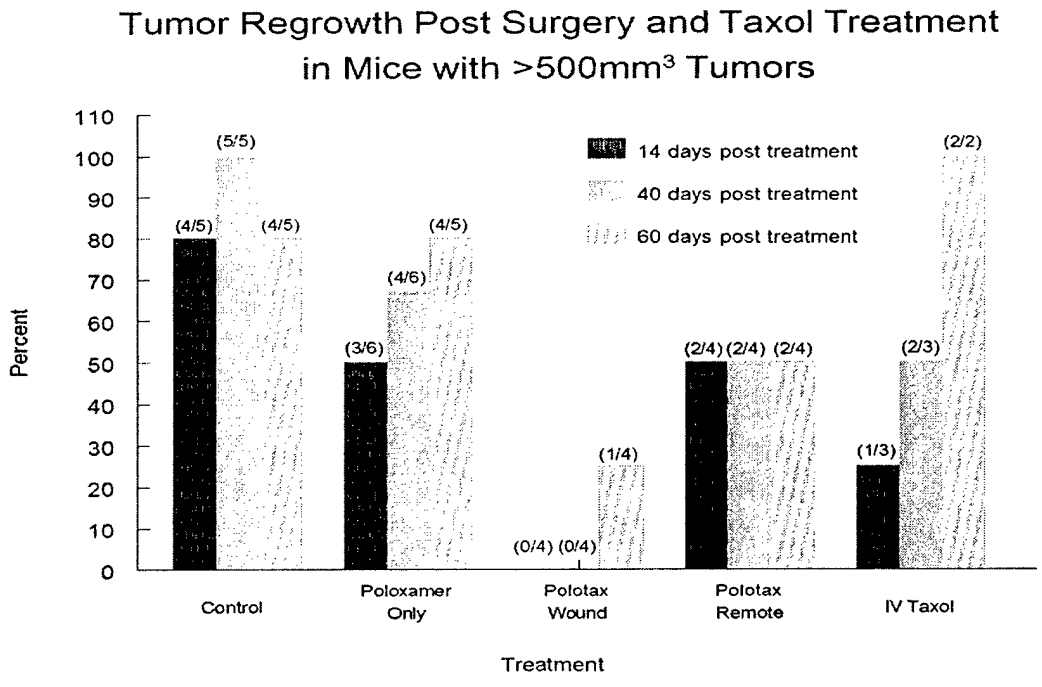


Figure 7
Tumor metastasis of MCF-7-Adr human breast tumor cells following marginal resection of local tumors in nude mice after postoperative treatments (to 40 days following treatment).

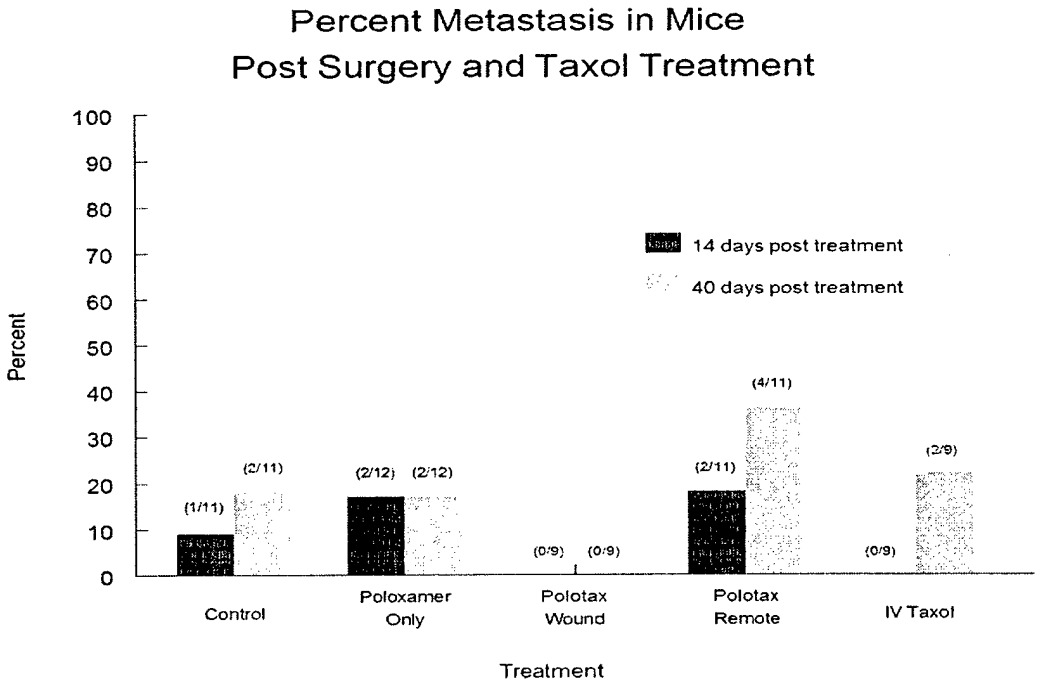


Figure 8A-C
Tumor metastasis of MCF-7-Adr human breast tumor cells following marginal resection of local tumors in nude mice after postoperative treatments based on size of the tumor at the time of surgical removal (to 60 days following treatment).

Figure 8A

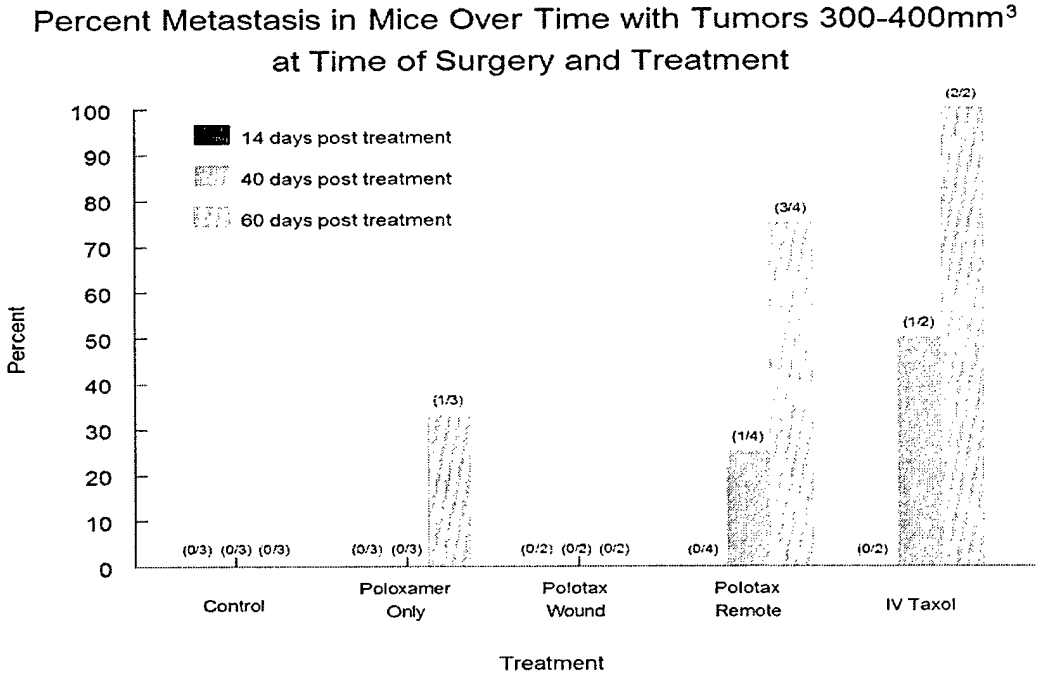


Figure 8B

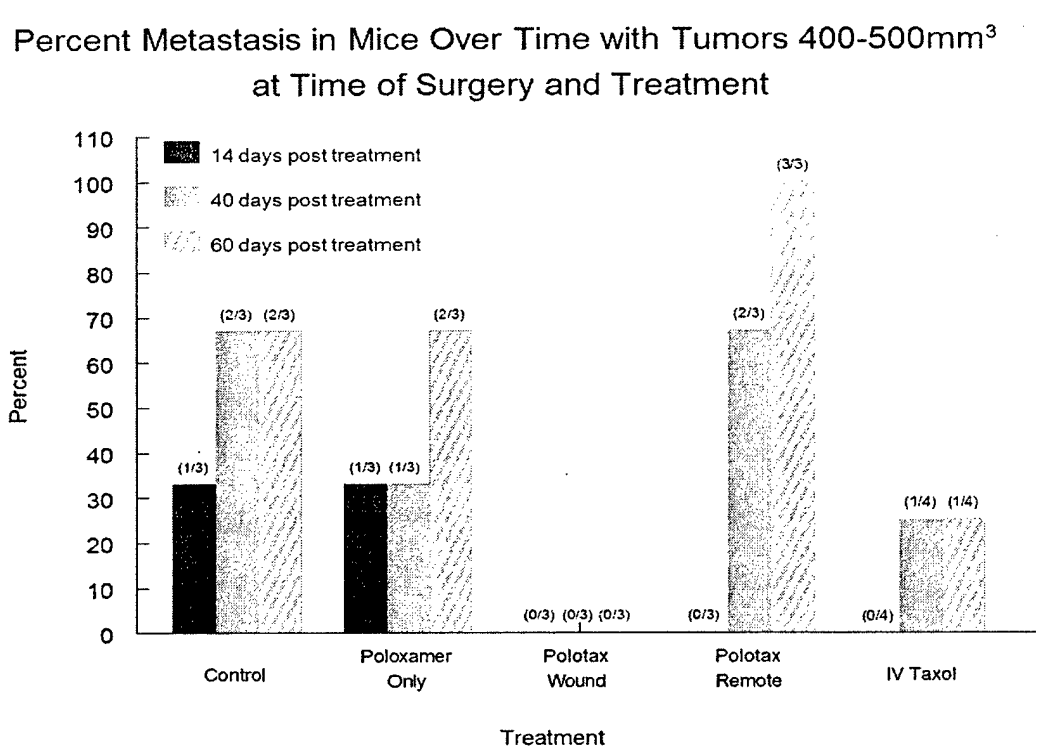
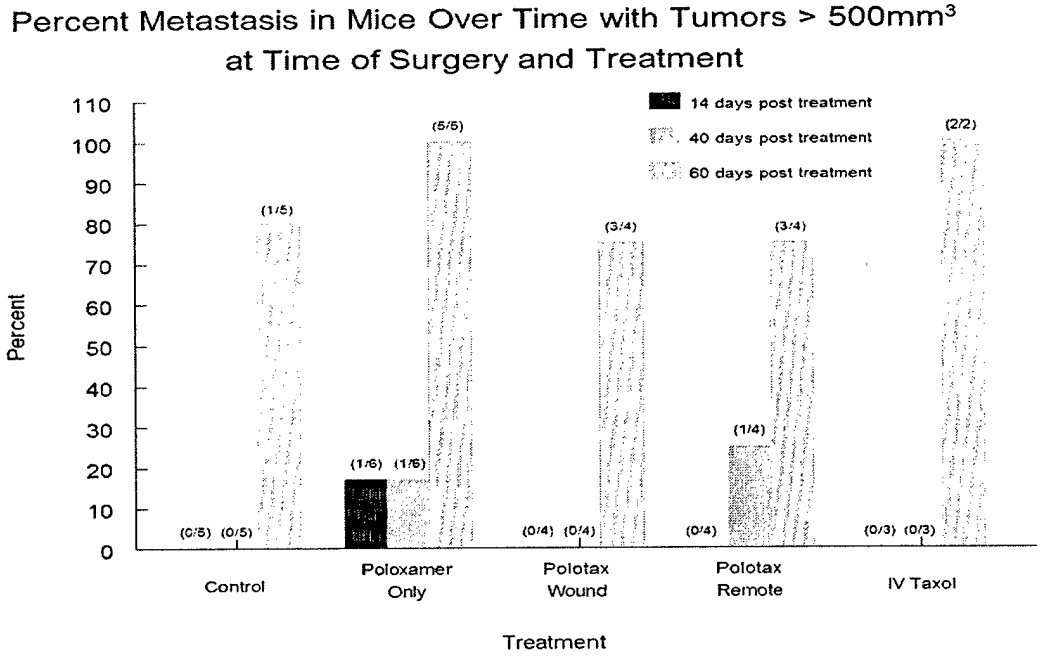


Figure 8C



Task 2

To evaluate the local and systemic toxicity of locally delivered (intracavitary; within the wound bed) paclitaxel chemotherapy following tumor removal.

Three of 12 mice treated with intracavitary polotax developed wound dehiscence and due to wound healing complications were sacrificed. Tissues have been obtained from all mice that have died or have been sacrificed and will be evaluated during year three for evidence of organ toxicity. Three of 12 mice treated with intravenous taxol died following injection from what appeared to be an anaphylactic reaction. This has been reported for taxol, specifically related to the carrier cremafor EL. Depended on histologic findings, We will attempt pretreatment of the mice prior to taxol injections using steroid and antihistamine to counteract this reaction.

Task 3

To evaluate the local (wound bed), regional (lymphatic) and systemic (organ system) distribution of paclitaxel following intracavitary polymer delivery

Tissues (and serum) will be obtained from all sacrificed, treated mice and banked for analysis of drug levels during year three of the grant.

Key Research Accomplishments:

1. Establishment of 5 (commercially available) human breast tumor cell lines within our laboratory.
    a. MCF-7
    b. MCF-7 AL
    c. MDA-MB-435

d. MDA-MB-231
e. MX1

2. In vitro cytotoxicity testing of these 5 cell lines for sensitivity to paclitaxel and polymer delivered paclitaxel.
3. Transfection of cell lines with the luciferase gene.
4. In vivo growth of MCF-7 AL cell line in nude mice.
5. Establishment of tolerability and efficacy of poloxamer locally delivered paclitaxel against MCF-7-ADR breast tumors in nude mice.

Reportable Outcomes:

Rizzo S, Hudacheck S, DeLille A, Dong B, Dernell W. 2004. *In vitro evaluation of the efficacy of polymer delivered paclitaxel chemotherapy against human breast tumor cell lines.* Poster presented at the **2004 Phi Zeta Research Day,** Colorado State University College of Veterinary Medicine and Biomedical Science, January 24, 2004, Fort Collins, CO.

Hudacheck S, Rizzo S, De Lille A, Dernell W. *Polymer taxol delivery in a mammary carcinoma model labeled for in vivo imaging.* Poster presented at the Annual **Biowest** Meeting, Aurora, CO, October, 2003.

De Lille A, Dernell W. *Luciferase in vivo imaging system: Applications.* Poster presented at the **University of Colorado Cancer Center** Poster Session, September, 2003.

Dernell, WS. *Evaluation of lymphatic drainage and uptake following intracavitary chemotherapy administration for mammary carcinoma.* 7/1/03-6/30/04. Grant submitted to the **US Army Medical Research and Materiel Command**. ***$75, 000*** *(direct costs).*

Conclusions:

1. Four of the five established human breast cancer cell lines established in our laboratory have shown sensitivity to paclitaxel.
2. Paclitaxel delivered through poloxamer has shown a synergistic effect on cytotoxicity for the chemotherapy resistant cell line, MCF-7-AR cell line. This synergism appears to be ATP dependent. Further research is warranted to evaluate (and substantiate) this mechanism.
3. Consistent growth has been established for the MCF-7-ADR cell line in nude mice, establishing this as a viable model for chemotherapy testing.
4. Poloxamer delivered paclitaxel has shown complete control of local tumor regrowth and metastasis following intracavitary treatment of marginally removed MCF-7-ADR tumors. This appears true for very large, established tumors as well as early, smaller growth.
5. Minimal control of local regrowth or systemic metastasis is seen following traditional intravenous administration of the parent drug, paclitaxel. The improved tumor control using poloxamer delivered paclitaxel (see 4 above) may reflect high local concentrations of drug as well as preferential uptake of drug/polymer within lymphatics; the first

direction of metastatic spread. Funding for further research on lymphatic uptake of poloxamer delivered paclitaxel is presently being sought.

6. Mild to moderate local tissue reaction is seen following intracavitary treatment of poloxamer delivered paclitaxel, without clinical evidence of systemic toxicity. Mechanisms to improve tissue tolerance of the paclitaxel/polymer warrant further investigation.
7. Marked systemic toxicity is seen following intravenous administration of the parent drug, paclitaxel. The reduced systemic toxicity using the poloxamer delivered paclitaxel offers a potential treatment advantage, especially if improved control of metastasis can be achieved.
8. The decision to obtain and utilize the in vivo luciferase imaging system has resulted the decreased use of animals and an increase in sensitivity of monitoring tumor progression and metastasis.

References:

Rizzo S, Hudacheck S, DeLille A, Dong B, Dernell W. 2004. *In vitro evaluation of the efficacy of polymer delivered paclitaxel chemotherapy against human breast tumor cell lines.* **Proceedings of the 2004 Phi Zeta Research Day:**13.

Sweeney TJ. Mailander V. Tucker AA. Olomu AB. Zhang W. Cao Y. Negrin RS. Contag CH. *Visualizing the kinetics of tumor-cell clearance in living a*nimals. **Proceedings of the National Academy of Sciences of the United States of America**. *96(21):12044-9, 1999.*

Vooijs M. Jonkers J. Lyons S. Berns A. *Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice.* **Cancer Research**. *62(6):1862-7, 2002.*

Appendix: N/A