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Award Number: W81XWH-04-1-0453

TITLE: Optimized NSAIDs for Breast Cancer Prevention

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REPORT DATE: April 2006

TYPE OF REPORT: Annual

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 contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-04-2006	Annual	26 Mar 2005 – 25 Mar 2006

4. TITLE AND SUBTITLE

Optimized NSAIDs for Breast Cancer Prevention

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0453

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dennis A. Carson, M.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California San Diego
La Jolla, CA 92093

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Population studies have shown that women who use non-steroidal anti-inflammatory drugs (NSAIDs) developbreast cancer less frequently. However, these drugs have side effects toward the stomach, liver and kidneys,particularly at the high doses potentially required to prevent breast cancer. Also, how these agents prevent breastcancer is not understood. This project will develop an optimized NSAID for breast cancer prevention that can betaken safely at high doses, and will determine its mechanisms of action. The side effects of NSAIDs are mainly dueto inhibition of cyclo-oxygenase (COX) enzymes. Based on preliminary experiments, we hypothesize that thepreventative action of NSAIDs in breast cancer is not solely due to COX inhibition, but rather to alterations of otherbiochemical pathways in breast cells that control their proliferation. We have isolated modified NSAIDs that do notinhibit the COX enzyme, but still retain chemopreventative activity. Testing this COX independent NSAID in arobust model of breast cancer, the MMTV-wnt1 transgenic mouse, has revealed a trend towards tumor preventionand a significant reduction in gene expression of wnt regulated targets. These data have already encouragedearly, biomarker based, clinical trials in women with breast cancer.

15. SUBJECT TERMS
Wnt, oncogene, transgenic mice

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

There is an unmet need for agents that can prevent the development of estrogen receptor negative breast cancer. A characteristic feature of these tumors is the high levels of expression of cyclin D1, that is an established target of the wnt oncogenic pathway. Indeed, MMTV-wnt1 transgenic mice develop breast cancer, in the absence of a functional estrogen receptor. R-etodolac, a non-steroidal anti-inflammatory drug devoid of the gastrointestinal side effects of cyclo-oxygenase inhibition, has been found to inhibit the wnt pathway in cell culture and *ex vivo* experiments. The ongoing experiments detailed in this report provide evidence for R-etodolac's activity *in vivo* in pre-malignant breast tissue. The work is innovative, because no safe inhibitors of wnt signaling are currently known. The results are clearly relevant to the needs of women at risk for breast cancer given that these data have already supported successful applications to fund clinical trials of this agent in combination therapy.

Body

Task 1. To determine if supplementation with R-eto can prevent the development of breast cancer in the MMTV-wnt1 trangenic model.

a. Increase size of breeding colony of FVB/NJ-TgN[Wnt]1 Heu mice, and confirm expression of the transgene in at least 50 female offspring (Months 1-6).

To date 102 MMTV-wnt1 positive transgenic females have been bred and randomized to receive R-eto or control chow.

b. Divide mice into drug-treated and control groups, and observe for breast cancer development (Months 7-17).

Observation for breast tumor development is complete. Sixty mice (31 control and 29 R-Etodolac feed) developed tumors (see *Figure* , Kaplan-Meier graph.) Although a trend towards chemoprevention was seen, this did not reach statistical significance ($p = 0.34$.)

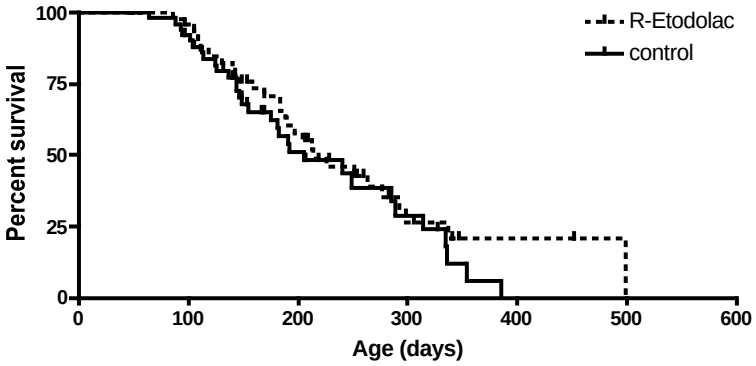

Figure 1. *Kaplan-Meier Survival Curves for MMTV-wnt1 transgenic mice on control versus R-Etodolac Chow.*

c. Perform pathologic and molecular analyses of breast tumors from mice in both groups (Months 12-20).

Pathologic analysis of breast tumors from MMTV-Wnt1 mice feed control chow or R-etodolac (1.25g/kg) chow revealed no gross morphologic differences. Hematoxylin and eosin (H&E) stained sections of paraffin embedded tumors from both groups revealed no significant differences (see figure 2 for representative images.)

Figure 2. *H&E stained sections of tumors from control (left panel) and R-etodolac treated (right panel) MMTV-wnt1 transgenic mice*

Molecular analysis of breast tumors from both groups revealed a trend towards Wnt1 inhibition with treatment. Quantitative PCR to measure gene expression was independently performed in triplicate on tumors from 5 mice from each group (see figure 3.) On average gene expression of Cyclin D1, the canonical wnt1 regulated target, was decreased by 33%. However due to tremendous inter-mouse variability the standard error was large and crosses unity.

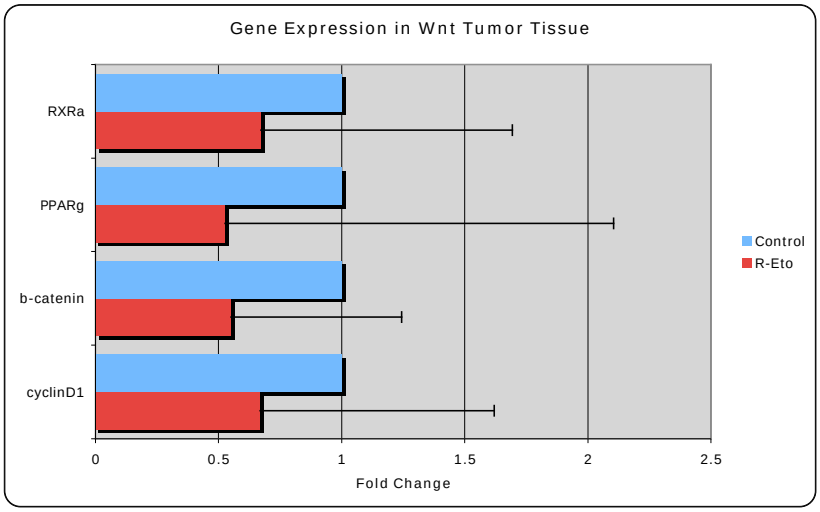

Figure 3. *Quantitative PCR for R-etodolac targets (RXRa and PPARg) and Wnt signaling pathway targets (b-catenin and Cyclin D1) reveals a trend towards decreased gene expression with R- Etodolac treatment (red bars) compared with control (blue bars.)*

d. Repeat treatment protocol in independent groups of mice, with dosage modification if indicated by first experimental data set (Months 21-30).

Based on the measurable alteration in the hypothesized target there is no need for repeat experiments in independent groups of mice. Additionally ongoing experiments in human subjects, spurred by this work, will provide the most robust possible data to further advance this novel agent.

e. Repeat analyses of excised breast tumors (Months 31-33).

Task not due at this time. Additionally, as per section 1d, there is no need for repeat experiments in independent groups of mice.

f. Complete supportive biochemical and pharmacologic studies (Months 34-36).

Task not due at this time.

Task 2. To determine the mechanism of action of the modified non-steroidal anti-inflammatory drug R-eto in mammary tissues, emphasizing the regulation of wnt signaling.

a. Optimize protocol for quantitative gene expression of wnt-related genes in primary mammary tissues (Months 1-6).

Task complete, see annual report from April 2005 for details.

b. Optimize immunohistochemical procedures for assessing wnt-signaling in primary tumors (Months 1-6).

Task complete, see annual report from April 2005 for details.

c. Complete analyses of gene and protein expression in tumors from MMTV-wnt1 trangenic mice (Months 7-36).

Given the large standard error seen in the quantitative PCR from MMTV-wnt1 tumors, attention was focused on the pre-malignant breasts from the treated and control groups. Again decrease gene expression was seen (see figure 4) in both R-etodolac targets (RXRa and PPARg) and downstream of these in the wnt signaling pathway (b-catenin and Cyclin D1.) However in this case the standard error, again with 5 mice in each group, was much narrower and did not cross unity, strongly supporting this finding.

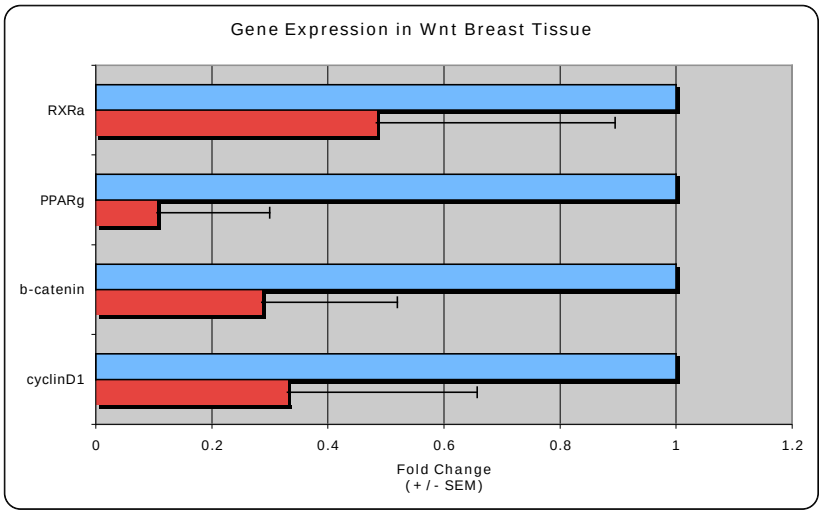

Figure 4. *Quantitative PCR for R-etodolac targets (RXRa and PPARg) and Wnt signaling pathway targets (b-catenin and Cyclin D1) reveals decreased gene expression with R- Etodolac treatment (red bars) compared with control (blue bars.)*

Whole mounts of this pre-malignant breast tissue (representative images shown in figure 5) reveal no significant change in duct morphology. This supports the hypothesis that R-etodolac is impacting Wnt signaling at the molecular level, rather than altering tissue architecture.

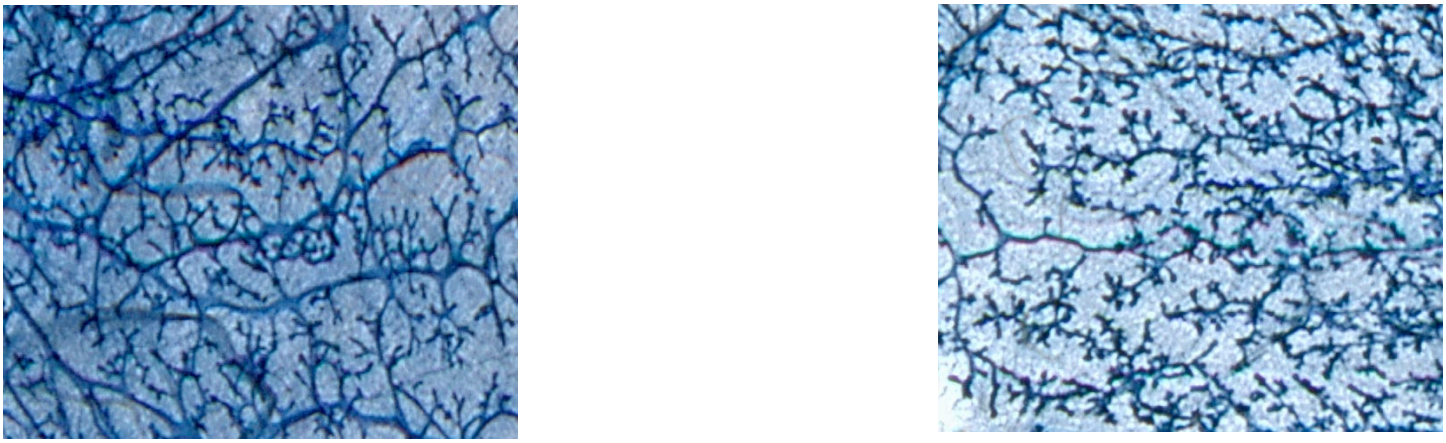

Figure 5. *Whole mounts of pre-malignant breasts from MMTV-Wnt1 mice on control (left panel) and R-etodolac (right panel) chow.*

d. Complete wnt signal transduction studies in breast cancer cells treated with R-eto and other NSAIDs (Months 1-36.)

Task not due at this time. Wnt signal transduction studies are being optimized using a western blot assay for Cyclin D1, the canonical downstream Wnt target. This assay will be used for both breast cancer cell line based studies and to confirm the findings in pre-malignant breast and breast tumors from MMTV-Wnt1 transgenic mice (see figure 6.)

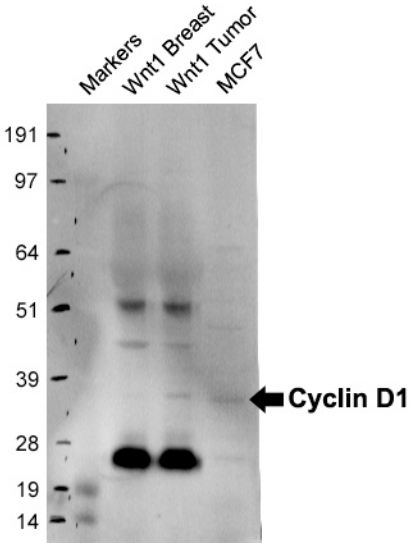

Figure 6*. Western blot for Cyclin D1 (33 kDa predicted molecular weight) using mouse monoclonal antibody from BD Pharmingen (Cat No 554180.) MMTV-Wnt1 pre-malignant breast tissue (labeled Wnt1 Breast) reveals only a faint band while both MMTV-Wnt1 breast tumor (Wnt1 Tumor) and a breast cancer cell line (MCF7) reveal stronger bands.*

Key Research Accomplishments

- A trend towards breast tumor prevention is seen with R-etodolac treatment with a modest sized cohort of MMTV-wnt1 transgenic mice, a robust model of breast cancer.
- Oral treatment with R-etodolac alters gene expression in pre-malignant breast tissue from MMTV-wnt1 transgenic mice.
- Data from these experiments have directly lead to clinical trials testing etodolac (a racemic mixture of R-Etodolac and S-Etodolac) in women with breast cancer.

Reportable Outcomes

Abstract
An abstract describing early immunohistochemical findings from these studies has been published [1].

Presentations
August 15th, 2005 – Preliminary data from these experiments were presented at an educational seminar at Celgene San Diego introducing the utility of biomarker based studies to assess novel therapeutic agents.

November 4th, 2004 – Preliminary data from these experiments were presented at Basic and Translational Research Rounds (BTRR) at the Moores UCSD Cancer Center. The BTRR series is designed to bring post-doctoral trainees and junior faculty from both basic and clinical research endeavors together to stimulate translational research.

February 24th, 2006 – An M.D. postdoctoral fellow presented preliminary data from these experiments during his faculty recruitment talk at the Moores UCSD Cancer Center.

April 13th, 2006 – This M.D. postdoctoral fellow presented preliminary data from these experiments during his faculty recruitment talk at Tufts University/New England Medical Center.

Funding applications
Based on preliminary data from these studies successful grant applications have been submitted to the Breast Cancer Research Foundation and the Amgen Oncology Institute Hematology and Oncology Fellowship Program with a combined budget of approximately $240,000. These funds are being used for translational projects testing racemic etodolac (including R-etodolac) in women with breast cancer. A K23 award based on preliminary data from the DOD project and the subsequent clinical studies has been submitted to NIH and is currently pending review.

Conclusions
These studies are based on the hypothesis that a COX-inactive NSAID (R-etodolac) can specifically block the wnt oncogenic pathway in breast cancer, without host toxicity. Evidence has continued to mount that the wnt signaling pathway is critical in breast cancer with the discovery that wnt signaling increases the number of breast stem cells[2]. Preliminary data reveals downregulation of wnt signaling and a trend towards reduction in breast tumor formation with R-etodolac treatment. These studies have taken on even greater importance as similar efforts to develop minimally toxic medications to reduce the risk of ER negative breast cancer have failed. In particular, early studies of COX2 inhibitors as chemopreventative agents while promising[3] have been halted due to cardiac toxicity[4-6]. The current studies could provide evidence to bring a COX-inactive NSAID with chemopreventative activity into large-scale clinical trials for women at risk of developing ER negative breast cancer.

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Appendices

None