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14. ABSTRACT
Population studies have shown that women who use non-steroidal anti-inflammatory drugs(NSAIDs) develop breast cancer less frequently. However, NSAIDs have side effects on thestomach and kidneys, particularly at the high doses potentially required to prevent breastcancer. This project has focused on developing an optimized NSAID for breast cancerprevention that can be taken safely at high doses, and determining its mechanisms of action.The side effects of NSAIDs are mainly due to inhibition of cyclo-oxygenase (COX) enzymes.Based on preliminary experiments, we hypothesized that the preventative action of NSAIDs inbreast cancer is not solely due to COX inhibition, but rather to alterations in the Wntsignaling pathway. Using a modified NSAID that does not inhibit the COX enzyme, but doesinhibit Wnt signaling, we attempted chemoprevention of breast tumors in the MMTV-wnt1 andMMTV-neu transgenic mouse stains. Significant gene expression changes in a Wnt targetinvolved in cancer proliferation, Cyclin D1, have been found. Unfortunately protein levelsof Cyclin D1 were unaffected and current experiments are characterizing the mechanism of thisdisparate finding. Regardless, these data have already encouraged early, biomarker based,clinical trials in women with breast cancer.

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Wnt, oncogene, transgenic mice

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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 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 Neu mice, and confirm expression of the transgene in at least 50 female offspring (Months 1-6).

As reported in our 2006 annual report 102 MMTV-wnt1 positive transgenic females were bred and randomized to receive R-etodolac or control chow.

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

As reported in our 2006 annual report observation for breast tumor development is complete. Sixty mice (31 control and 29 R-Etodolac feed) developed tumors (see *Figure 1*.) Although a trend towards chemoprevention was seen, this did not reach statistical significance ($p = 0.34$.)

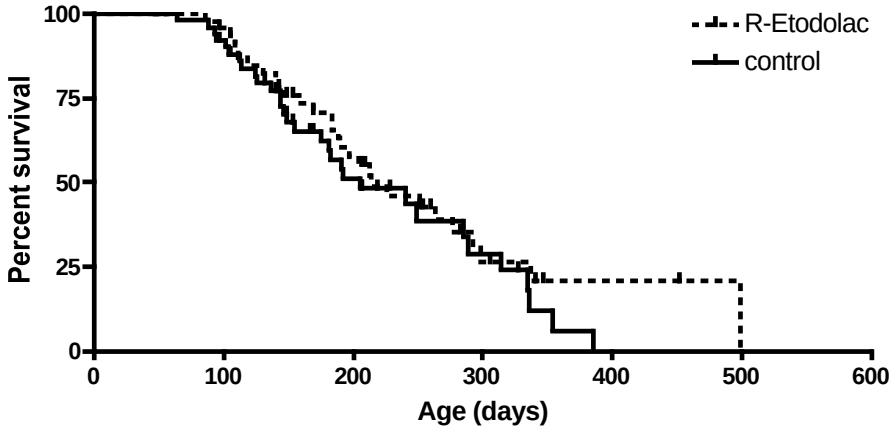

Figure 1. *Kaplan-Meier Survival Curves for MMTV-wnt1 transgenic mice on control versus R-Etodolac Chow.*

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

As reported in our 2006 annual report 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 this decrease was not statistically significant for any tested gene.

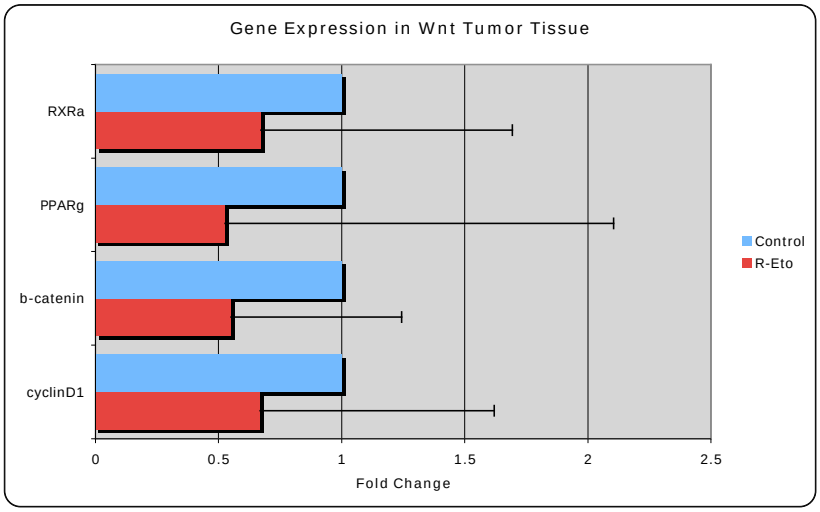

Figure 3. *Quantitative PCR for R-etodolac targets (RXRa and PPARg) and Wnt signaling pathway targets (b-catenin and Cyclin D1) reveals a trend towards decreased gene expression with R- Etodolac treatment (red bars) compared with control (blue bars.)*

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

Higher doses of R-etodolac are not tolerated in mice. Therefore an independent group of mice with an alternative breast cancer inducing transgene, MMTV-Neu [exact stain name, FVB/N-Tg(MMTVneu)202Mul/J] were breed. Fifty-nine female mice were breed and randomized to either R-etodolac (1.25g/kg) or control chow. Although an early trend towards chemoprevention of breast tumors was seen (see figure 4), at the termination of the experiment the difference in breast tumor formation was not statistically significant (p=0.47.)

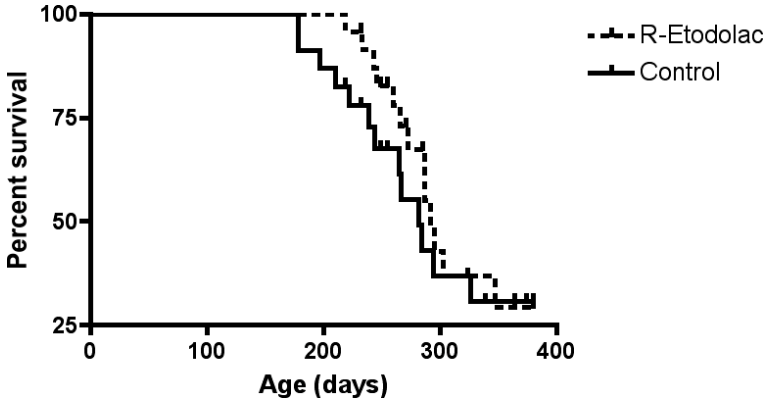

Figure 4. *Kaplan-Meier Survival Curves for MMTV-wnt1 transgenic mice on control versus R-Etodolac Chow.*

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

Hematoxylin and eosin (H&E) stained sections of paraffin embedded tumors from both groups of FVD-Neu mice revealed no significant histologic differences. However a significant reduction in Cyclin D1 gene expression is seen with R-eto treatment (see figure 5.)

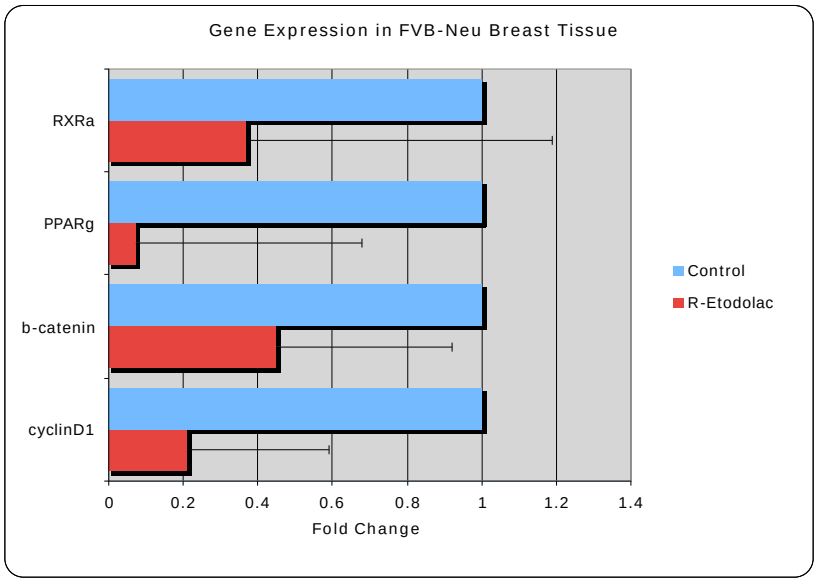

Figure 5*. Quantitative PCR for R-etodolac targets (RXRa and PPARg) and Wnt signaling pathway targets (b-catenin and Cyclin D1) reveals decreased gene expression with R- Etodolac treatment (red bars) compared with control (blue bars) for certain genes. For Cyclin D1 this reduction (79%) is statistically significant (p=0.037) while a large reduction in PPARg (91%) only demonstrates a strong trend towards significance (p=0.074) due to assay variability.*

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

Supporting biochemical experiments were conducted using standard MTT viability assays on cells treated with R-eto for 72 hours, the half maximal inhibitory concentration (IC50) was determined. For the classic "normal" breast cell line MCF-7 an IC50 of 625 micromolar was determined. For MCF-7 cells made resistant to the standard chemotherapeutic agent adriamycin, the IC50 exceeded 1000 micromolar.

Supporting pharmacologic studies on plasma from treated and control mice were performed using high pressure liquid chromatography (HPLC.) Treated mice had a mean R-eto plasma level of 286 micromolar while control mice had no detectable R-Eto. See figure 6 for calibration curves using two alternate UV detection wavelengths for quantification. The results of both techniques closely correlate and are averaged.

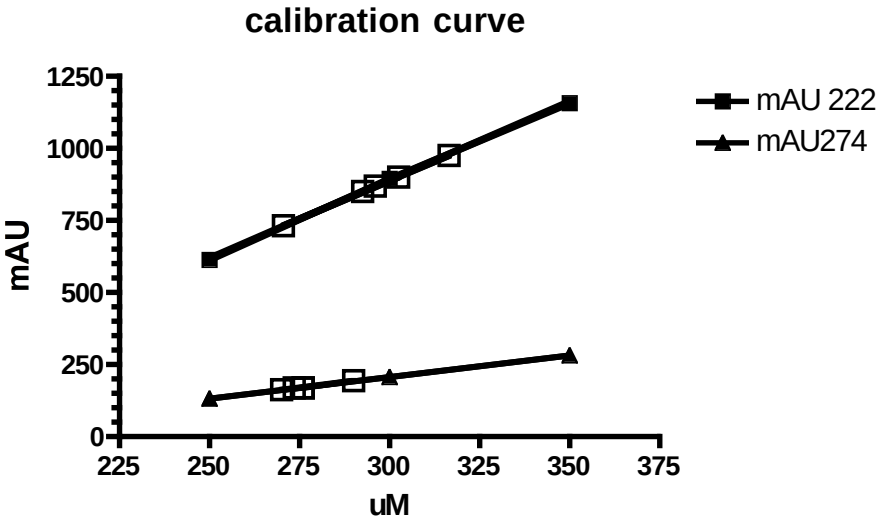

Figure 6. *HPLC calibration curves with plasma R-eto concentrations plotted for 5 mice given 1.25mg/kg R-Eto containing chow ad libitum. Averaging the similar results of measuring at two alternate wavelengths reveals a plasma concentration of 286.33 +/- 5.2 micromolar.*

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).

As we reported in our April 2005 annual report;

A commercially available kit, the Qiagen RNeasy Kit, was used to extract RNA from flash frozen Wnt1 breast tumors from control and treated mice. Tumors were pulverized with mortar and pestle while maintained at less than –80 degrees Celsius prior to extraction. Purified RNA was utilized in Affymetrix gene chip arrays through the UCSD Cancer Center Genechip Core. Table 1 details preliminary results of this experiment.

Table 1 – Change in gene expression with R-Eto treatment.

Fold reduction
11 - Casein alpha
10 - Protein Phosphatase 1, catalytic subunit, beta isoform
8 - Peroxiredoxin 1
7 - Bcas1
7 - Acid phosphatase 1, soluble

Fold Induction
17.5 - Major urinary protein 1
11 - Collagenous repeat-containing sequence
11 - Retinitis pigmentosa GTPase regulator interacting protein 1
8 - Adipsin
8 - Adipocyte complement related protein of 30 kDa (Acrp30)
6 - Carbonic anhydrase 3
6 - Fatty acid binding protein 4

Table 1 – Comparison of gene expression between MMTV-wnt1 breast tumors developing in mice on control versus R-Eto chow.

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

As we reported in our April 2005 annual report;
Antibodies directed against Wnt1 are not effective in immunohistochemistry. This is generally believed to be related to Wnt1's tight association with extracellular matrix. Therefore to assess wnt-signaling, immunohistochemical assays for a known downstream target (Cyclin D1), newly identified drug targets (PPARγ and RXR[1]), and a standard marker of proliferation (Ki67) were optimized in breast tumors from treated and untreated MMTV-wnt1 transgenic mice. Tumors were formalin-fixed and paraffin-embedded prior to sectioning to avoid sectioning artifact, a common problem in tissues with high fat content. Sections were then deparaffinized by a standard procedure and antigen retrieved with DAKO antigen retrieval solution[2]. Polyclonal antibodies that react with murine antigens were used for each of the following assays; anti-PPARγ antibody (ABCAM 12410), anti-RXRα antibody (Santa Cruz Biotechnology D20), anti-Ki67 antibody (Novocastra NCL-Ki67p) and anti-cyclin D1 antibody (biosource AHF0102.) Preliminary results revealing chemomodulation PPARγ and RXRα along with downregulation of Cyclin D1 and Ki67 are shown in figure 7.

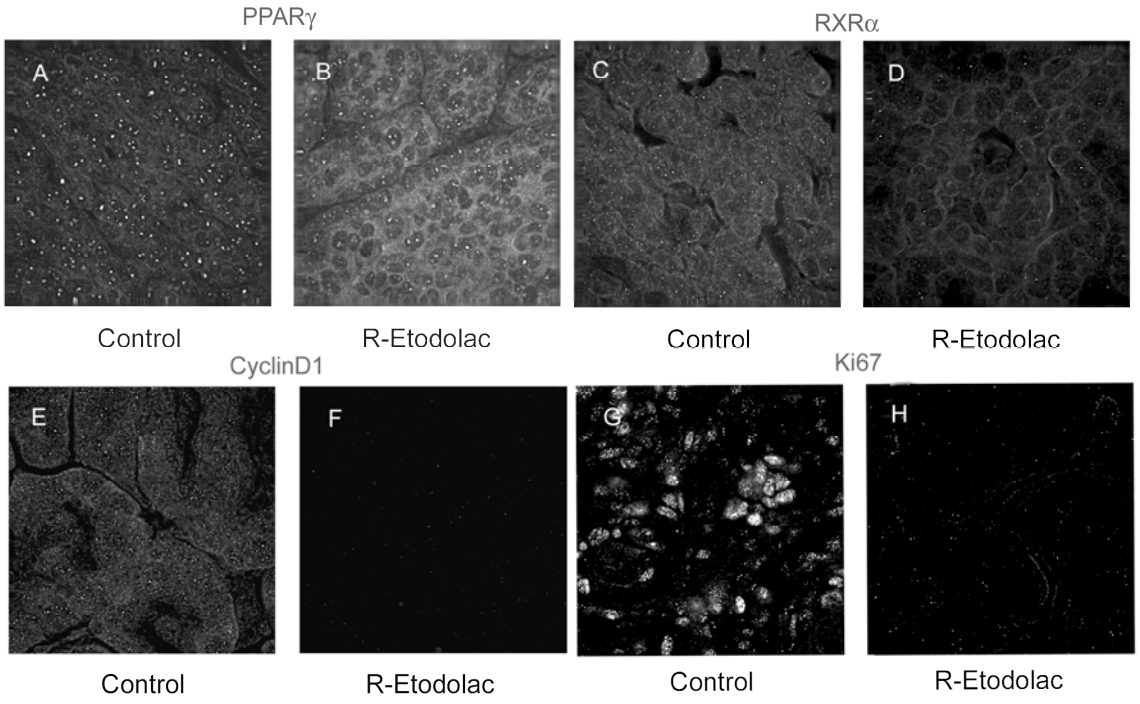

Figure 7- Immunohistochemical stain of breast tumors from MMTV-wnt1 transgenic mice. Transgenic MMTV-wnt1 mice that developed nodules while fed control versus R-etodolac supplemented chow. The mice were sacrificed and the tumors were excised, fixed and embedded. Histologic sections were stained with the indicated antibodies. Images were taken using a DeltaVision Deconvolution Microscope at a magnification of 400X**.**

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 8) 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, but without reaching statistical significance.

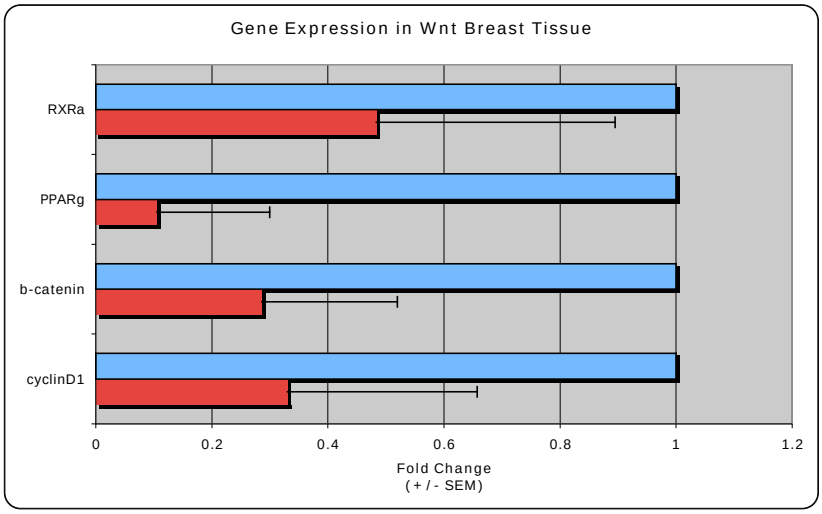

Figure 8. *Quantitative PCR for R-etodolac targets (RXRa and PPARg) and Wnt signaling pathway targets (b-catenin and Cyclin D1) reveals decreased gene expression with R- Etodolac treatment (red bars) compared with control (blue bars.)*

Whole mounts of this pre-malignant breast tissue (representative images shown in figure 9) 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.

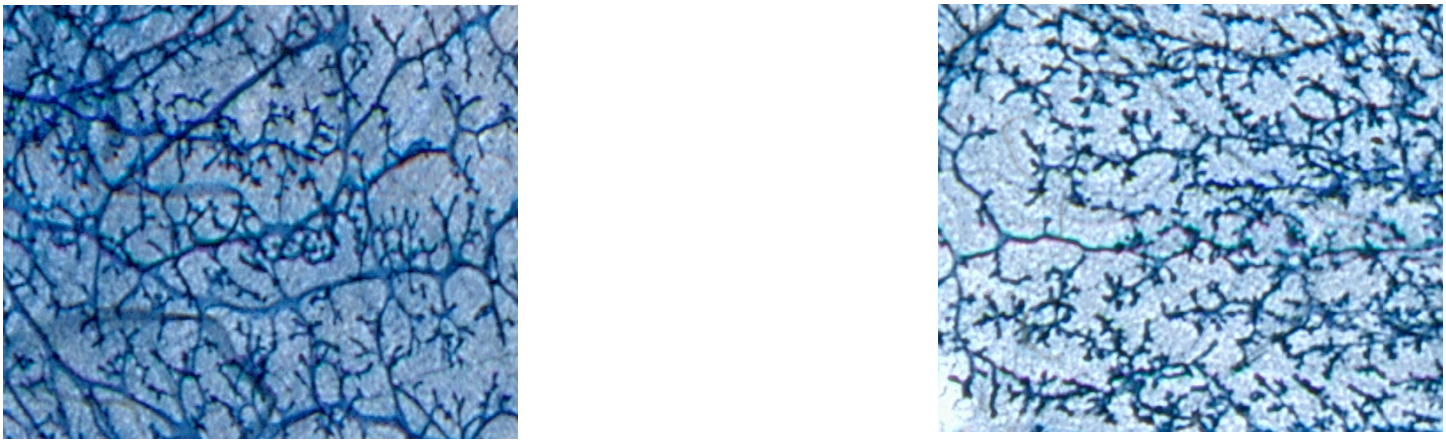

Figure 9. *Whole mounts of pre-malignant breasts from MMTV-Wnt1 mice on control (left panel) and R-etodolac (right panel) chow.*

Western blot assays (figure 10) for the Wnt1 target protein most strongly suppressed at the gene expression level, Cyclin D1 in FVB-Neu mouse pre-malignant breast, revealed no consistent reduction in protein level. Similarly no consistent reduction in beta-catenin protein levels are seen (an alternate Wnt1 target.)

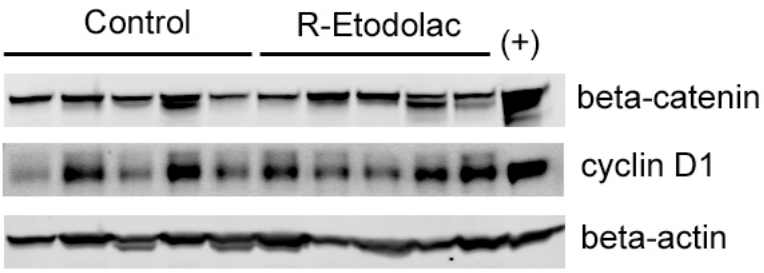

Figure 10 *Protein lysates extracted from flash frozen pre-malignant breast from FVB-Neu mice were analyzed by standard western blotting. Lysates from mice treated with 1.25mg/km R-Eto containing chow ad libitum reveal no consistent difference for beta-catenin or cyclin D1 protein level compared with mice on control chow. Significant mouse to mouse variability is noted in both treated and control mice.*

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

An exhaustive *in vitro* study of Wnt/beta-catenin signaling inhibition was performed using all available NSAIDs (see table 2.) R-eto performed extremely well compared to other, COX inhibitory NSAIDs, even though R-Etodolac has a dramatically improved toxicity profile compared with the COX inhibitory NSAIDs.

Table 2. Inhibition of β-catenin/PPARγ/RXRα signaling by NSAIDs

Drugs	Inhibition rates (%)				
	26μM	52μM	104μM	208μM	416μM
R-etodolac	96.3±4.5	85.2±1.2	76.2±2.4	58.5±3.1	35.6±1.1
Acetylsalicylic acid	101.8±0.8	96.9±1.4	96.3±0.6	92.3±0.4	89.1±4.8
Naproxen	112.8±0.4	106.9±0.4	100.3±7.7	90.7±0.9	55.2±2.4
Ketoprofen	101.0±4.9	88.5±2.4	79.4±2.4	65.3±2.9	53.1±3.8
Ketorolac	97.5±1.1	94.5±1.1	98.7±3.2	78.5±0.3	47.9±1.1
Ibuprofen	103.7±2.6	97.6±3.9	78.1±2.9	62.8±0.4	50.3±0.3
Piroxicam	116.6±7.4	114.0±5.4	101.9±3.4	94.4±2.8	88.4±3.8
Meloxicam	102.8±3.5	118.7±4.7	98.3±0.0	93.8±10.6	78.6±3.6
Salsalate	99.6±10.0	85.5±1.2	74.4±4.6	53.8±4.9	43.6±3.9
Flurbiprofen	129.7±3.6	121.0±4.7	109.8±0.1	79.0±1.3	41.7±1.5
Sulindac	106.7±7.9	90.9±5.1	62.7±6.4	41.3±0.3	28.5±3.9
Tolmetin	95.6±8.1	83.1±7.7	85.3±0.6	67.7±1.7	53.8±0.3
Fenoprofen	94.3±2.6	95.0±1.1	75.3±5.2	50.6±0.1	30.3±1.4
Diflunisal	97.9±2.4	90.9±6.7	75.2±4.5	58.0±0.3	18.9±0.2
Diclofenac	108.5±12.8	95.3±3.2	84.1±5.6	53.8±4.6	9.1±0.1
Indomethacin	56.0±0.1	46.2±1.2	41.2±0.4	28.8±1.3	5.2±0.4
Meclofenamic acid	108.4±1.3	99.0±2.1	82.0±1.3	41.4±1.3	13.1±0.2
Nabumetone	141.1±5.8	138.5±4.6	101.2±1.4	52.8±6.6	24.9±1.0

Results are mean ± SD, Values represent percentages relative to the control value (DMSO treatment = 100%).

Key Research Accomplishments

- Oral treatment with R-etodolac significantly decreases Cyclin D1 gene expression in pre-malignant breast tissue from MMTV-Neu transgenic mice.
- Similar changes in protein expression are not seen for Cyclin D1.
- Ongoing experiments to identify the molecular mechanism of disparate gene and protein expression may provide a critical piece of knowledge towards the use of R-etodolac as a chemopreventative agent.
- 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 – An 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 $490,000. These funds are being used for translational projects testing racemic etodolac (including R-etodolac) in women with breast cancer.

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]. Our DOD funded experiments reveal downregulation of wnt signaling at the gene level in a robust mouse model of breast cancer without significant alteration in wnt target protein levels. Based on these results we are performing ongoing experiments to identify the mechanism of disparate regulation and resistance.

This work has 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 complete studies provide a critical step towards developing a COX-inactive NSAID with chemopreventative activity.

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Appendices

None