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TITLE: Pim-1: A Molecular Target to Modulate Cellular Resistance to Therapy in Prostate Cancer

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The contract supports studies to define the role of the PIM1 kinase in acquired resistance to chemotherapy by prostate cancer cells. Data to date for specific aim #1 define a signaling pathway induced by docetaxel, involving sequential steps of STAT3 activation, expression of PIM1, and activation of NFkB signaling. Blockade of this pathway by expression of dominant negative PIM1 proteins blocks drug-induced upregulation of NFkB activity, and sensitizes cells to docetaxel. Other studies (specific aim #2) focus on identifying a mechanism through which PIM1 activates NFkB. We have unambiguously identified S937 as the major PIM1 phosphorylation site on the NFkB1/p105 precursor protein, through use of LCM/MS/MS analysis. Interestingly PIM2 is only a weak kinase for this site. Additional data (specific aim #3) have been generated to characterize a small molecule inhibitor of PIM1.
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INTRODUCTION
Studies under this funded activity are focused on characterizing the role of the PIM1 gene in acquired resistance to chemotherapy drugs, by prostate cancer cells. The proposal included three specific aims: 1) to define a novel signal transduction pathway activated by docetaxel, 2) to characterize the mechanism through which PIM1 activates and regulates NFkB signaling, and 3) to explore genetic and pharmacologic means of inhibiting PIM1 activity or expression to enhance the sensitivity of prostate cancer cells to docetaxel and other chemotherapy drugs. Substantial progress has been made in each of these areas during the 01 year of support.

BODY
We will outline our progress through reference to the specific aims described above. The first specific aim was to outline a signal transduction pathway activated by docetaxel and involving upregulation of PIM1 expression. This pathway has been substantially defined. Using RWPE1 and RWPE2 (not shown) prostate cells, we noted that docetaxel treatment rapidly leads to an increase in expression of the PIM1 serine/threonine kinase. Expression becomes apparent at 3hrs after drug addition, peaks at 9-12hrs, and returns to baseline by 24hrs (Fig. 1). This increase in expression is accompanied by an increase in pim-1 mRNA, as shown by real time-PCR analysis (Fig. 2). Thus the effects of docetaxel are primarily transcriptional or post-transcriptional.

We next wanted to define mechanisms through which pim-1 could be transcriptionally upregulated. Transcription of pim-1 is known to be activated by STAT transcription factors and by NFkB transcription factors. We engineered RWPE2 cells to constitutively express a NFkB-dependent promoter/luciferase plasmid, and the time course of STAT3 activation after docetaxel treatment (Fig. 1), and noted that it paralleled the course of pim-1 expression. We therefore suspected that docetaxel increased pim-1 expression in a STAT3-dependent manner. This was directly demonstrated by use of decoy oligonucleotides (Fig. 3). Double-stranded DNA oligonucleotides matching a known STAT3 binding site blocked the drug-induced upregulation of pim-1 expression, while a decoy based on a mutated (non-binding) STAT3 site did not. These data therefore establish a linear relationship among the following events: docetaxel treatment → STAT3 activation → pim-1 expression.

We hypothesized that NFkB transcriptional activation would be a downstream event in this signal transduction pathway, because many chemotherapy drugs and other stressors are known to activate NFkB. We engineered RWPE2 cells to constitutively express a NFkB-dependent promoter/luciferase plasmid, and
found that docetaxel treatment increased NFkB transcriptional activity. We then transiently infected these cells with a pim-1-encoding retrovirus. Pim-1 expression also consistently increased NFkB transcriptional activity (Fig. 4). To determine if the drug-induced increase in NFkB activity occurred in a pim-1-dependent manner, we then infected the reporter cell line with a retrovirus encoding a dominant-negative form of pim-1, pimNT81. The dominant negative pim-1 cDNA completely blocked the drug-induced upregulation of NFkB activity, demonstrating that pim-1 expression is a necessary upstream step in the drug-induced activation of NFkB (Fig. 5). In aggregate these studies establish a signal transduction pathway triggered by docetaxel treatment of RWPE2 prostate cancer cells.

To determine if this pathway modified drug toxicity, we examined the effects of enforced expression of wild-type or NT81 pim-1 cDNAs of docetaxel cell kill (Fig. 6). Docetaxel produced dose-dependent cell kill in RWPE1, 2 cells. Enforced expression of wild-type pim-1 cDNA markedly reduced cell death. In contrast, expression of the dominant negative NT81 cDNA enhanced cell death after docetaxel treatment. These data demonstrate that pim-1 expression can modulate drug-induced cell death, and demonstrate that the survival pathway described above is a legitimate target for pharmacologic intervention. These data will be presented at the 2006 AACR meeting in poster form (1).
The goal of specific aim #2 was to define pathways through which the PIM1 kinase could activate NFkB transcriptional activity. We had hypothesized that PIM1 would phosphorylate the NFkB1/p105 precursor protein on serine-937, leading to proteolytic cleavage of the protein with release of active p50 protein as well as other sequestered NFkB components and the TPL2 kinase. A major goal of this specific aim was to identify the phosphorylation site on p105. We have used a variety of biochemical methods to accomplish the unambiguous identification. We initially expressed the full-length p105 protein in bacteria. This was reacted in a variety of in vitro kinase reactions with recombinant PIM1 or PIM2 enzymes. PIM1 strongly phosphorylated p105, but only in the presence of manganese, not magnesium. PIM2 was a much weaker kinase (Fig. 7).

To demonstrate the site of phosphorylation we used mass spectroscopy of trypsin-digested fragments of p105 that had been phosphorylated in vitro. We had previously demonstrated that PIM1-dependent phosphorylation happens exclusively on serine. Fragments were separated by LC/MS/MS analysis and mass/charge rations were determined. The predicted peptide fragment that would result from phosphorylation at serine-937 was recovered, with a mass of 1016 (Fig. 8). Since there are several potential phosphorylation sites within this peptide, we proceeded to sequence the peptide with mass spectroscopy. Only the fragment corresponding to phosphoserine-937 was not recovered. These data unambiguously demonstrate that the major phosphorylation site of PIM1 on p105 is serine-937. We also found evidence by MALDI-MS that serine-851 may also be phosphorylated by PIM1. These data have not yet been confirmed by LC/MS/MS analysis.
The third specific aim proposed to use small molecule inhibitors of the PIM1 kinase as molecular probes to determine their effect on docetaxel sensitivity. We have submitted a report describing one such molecule, the flavonol quercetagetin (2). We have demonstrated that quercetagetin in a moderately potent (IC$_{50}$ = 340nM, specific, and cell-permeable inhibitor of PIM1 activity in prostate cancer cells. Key data include the demonstration that quercetagetin in competitive with ATP. A crystal structure of PIM1 in complex with quercetagetin, or with three other flavonoids, has been determined. We have also shown that quercetagetin is able to inhibit the activity of the PIM1 kinase in prostate cancer cells at an IC$_{50}$ of about 5.5μM. Interestingly the activity of the AKT kinase is not inhibited at all under these conditions (Fig. 9). RWPE2 prostate cancer cells treated with quercetagetin develop morphologic changes consistent with differentiation or senescence, accompanied by profound growth inhibition, at concentrations that inhibit PIM1 kinase activity (Fig. 10).

**KEY RESEARCH ACCOMPLISHMENTS**

- Definition of a novel survival pathway activated by docetaxel treatment, and involving sequential activation or expression of STAT3, PIM1, and NFκB components.
- Identification of serine-937 as the major phosphorylation site for PIM1 on the p105/NFκB1 precursor protein
- Identification of quercetagetin as a moderately potent and specific, cell-permeable PIM1 kinase inhibitor
- Abstract accepted for presentation at the annual AACR meeting, Washington DC, April, 2006
REPORTABLE OUTCOMES
None in 01 year

CONCLUSIONS
Our data demonstrate that PIM1 is a critical component of a survival/stress pathway activated by docetaxel treatment of prostate cancer cells. This pathway leads to activation of NFκB-dependent transcription, possibly by phosphorylation of p105/NFκB1 by PIM1 at serine-937. Targeting PIM1 kinase activity with quercetagetin, or other PIM1 kinase inhibitors, may lead to additive or synergistic cell kill following docetaxel treatment.

REFERENCES
1. Zemskova M, Sahakian E, Lilly M: The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death (abstract #2777), approved for presentation at 97th Annual Meeting of AACR, Washington, DC, April 2006.

APPENDIX

Research data are presented throughout the body of this report. The appendix contains two items:

1. AACR abstract #2777, approved for presentation at the 97th Annual Meeting, April, 2006, entitled “The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death” by M. Zemskova, E. Sahakian, M. Lilly.

2. Curriculum vitae for Michael Lilly, MD
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Updated 1/2006

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Education: Southern Adventist University, Collegedale, TN
1967-1971, B.A. (biology, chemistry)

Loma Linda University, Loma Linda, CA
1971-1975, M.D.

Postgraduate Training
Internal Medicine residency
University of Alabama at Birmingham
Birmingham, AL 1975-1978

Hematology-Oncology Fellowship
University of Alabama at Birmingham
1978-1981

Faculty Positions: 6/81-6/82 Instructor in Medicine
UAB School of Medicine

6/82-10/88 Assistant Professor of Medicine,
UAB School of Medicine

6/82-10/88 Associate Scientist, Lurleen Wallace
Tumor Institute, Birmingham, AL
Faculty Positions (cont’d):

6/89-9/98  Associate Professor of Medicine, University of Washington School of Medicine, Seattle, WA

4/96-10/96  Visiting Scientist, The Walter and Eliza Hall Institute for Medical Research, Melbourne, Victoria, AUSTRALIA

9/98 – present  Professor of Medicine & Microbiology Director, Center for Molecular Biology & Gene Therapy Loma Linda University School of Medicine, Loma Linda, CA

Hospital Positions:

1981-1988  Attending Physician, University of Hospitals and Clinic
Alaska
1981-1988  Staff Oncologist, Birmingham VA Medical Center, Birmingham, AL
1989-1998  Staff Oncologist, Seattle VA Medical Center, Seattle, WA
1998-present  Attending Physician, Loma Linda University Medical Center, Loma Linda, CA

Honors:

1974  Alpha Omega Alpha
1980  National Research Service Fellow
1981  Fellow, American College of Physicians

Board Certification:

1979  American Board of Internal Medicine
1980  ABIM Subspecialty Exam, Hematology
1981  ABIM Subspecialty Exam, Med. Oncology

Licensure:

Alabama Medical License #7730 (3/77-12/91)
Washington State License #27864 (12/91 – 12/00)
California Medical License #G84932 (12/98 – present)

Organizations:

Fellow, American College of Physicians
Member, American Society of Hematology
Member, American Society for Bone Marrow Transplantation
Member, American Society for Gene Therapy

National Professional Responsibilities

Member, ad hoc study sections for NIH:
1987  Diagnostic Radiology
1988  Experimental Therapeutics

Member, site visit team for program project
Dr. George Hahn, PI; Stanford University  
1988, 1989  
Member, site visit team for program project  
Dr. Bayard Clarkson, PI, Memorial-Sloan Kettering Inst., 1997

Special Local Responsibilities

Member, Scientific Review Subcommittee  

Member, Research & Development Committee  
SVAMC, 1994, 1995

Member, Hospice Advisory Committee  
SVAMC, 1994, 1995

Board Development Committee, Leukemia & Lymphoma Society (Southern California Chapter), 2003

Consultant

Cetus Corporation (1986)  
EncorePharma (2001-present)  
Myriad Genetics (2002-present)  
Exelixis Pharmaceuticals (2005-present)

GRANTS & CONTRACTS (PRINCIPAL INVESTIGATOR)  
Note: This listing does not include multicenter clinical trials in which Dr. Lilly was the local principal investigator.

National Institutes of Health  F32CA27980  Hyperthermia of animal and human tumors; 7/80-6/82

National Institutes of Health  R01CA18138-11  Prediction of thermal tolerance by in vivo NMR spectroscopy; 7/82-6/83

National Institutes of Health  R01CA36790  Assessment of hyperthermia by in vivo 31P-NMR spectroscopy; 9/84-9/87

Cetus Corporation  Characterization of a human granulocyte CSF; 7/85-6/86

National Institutes of Health  R01CA45672  Cytokine signaling in myeloid leukemia; 9/87-10/98

VA Merit Review Award  Non-protein hematopoietic agents; 10/90-4/97

March of Dimes Birth Defects Foundation  Characterization of a 28kd protein related to G-CSF; 7/93-6/96

Lymphoma Research Foundation of America  Mechanism of action of the pim-1 oncogene; 7/95-7/96
Roche Pharmaceuticals  

Preclinical study of Roferon and bryostatin 1 in a melanoma model; 1/98-12/99

Department of Defense, National Medical Technology Testbed #76-FY99: Cell-permeable proteins for cell regulation. 12/99 – 7/02

Leukemia Society of American Translational Award Propionic Acid Analogues for CLL. 9/1/01 – 8/31/05

Celgene Corporation, Phase I-II trial of combined GM-CSF (sargramostim) and thalidomide for hormone-refractory prostate cancer (5/02-5/04).

National Institutes of Health  R03CA107820 Molecular Targets of NSAIDs in Prostate Cancer; (5/1/04 – 4/30/07)

Department of Defense, CDMRP Prostate Cancer Program PC040635 Pim-1: A Molecular Target to Modulate Cellular Resistance to Therapy in Prostate Cancer (10/04 – 10/07)

Pharmion Corporation, Use of azacytidine to reverse silencing of GST-p1 in early prostate cancer. (10/05 – 10/07)

GRANTS and CONTRACTS (Co-investigator)

National Institutes of Health  R01CA097043 Molecular pathology of 2-deoxy-5-azacytidine; L. Sowers, PI; Michael Lilly, co-investigator (10% FTE). 7/1/03 – 6/30/08

PUBLICATIONS IN PEER-REVIEWED JOURNALS


33. Asiedu C, Biggs J, Michael B. Lilly, Kraft A: Inhibition of leukemic cell growth by the protein kinase C activator Bryostatin 1 correlates with the dephosphorylation of cyclin-dependent kinase 2. *Cancer Res* 55:3716-3720, 1995


BOOKS AND CHAPTERS:


RECENT ABSTRACTS:


Lilly M, Cooper JJ: Enforced expression of the human 33kd Pim-1 kinase prevents apoptosis-associated mitochondrial dysfunction and upregulates bcl-2 mRNA expression in murine myeloid cells. (oral presentation, ASH 12/97)


Chen CS, Lilly MB, Wang FS, Howard FD, Houwen B: Rapid monitoring of peripheral blood stem cells (PBSC) mobilization by using cell membrane phospholipid content correlates well with CD34+ measurements, successful harvest and engraftment (abstract #1642). *Blood* 96:380a (poster presentation, ASH 12/00)


Lilly MB, Wechter W, Puuvula L, Henry H: R-Flurbiprofen (RFB) a non-steroidal anti-inflammatory drug (NSAID) with anti-tumor activity, inhibits the expression of CYP24 in murine prostate carcinomas. (poster presentation at *Biennial Vitamin D Conference “Vitamin D and Cancer Chemoprevention”*, NIH, Bethesda, MD, November 2004)