AD_____

Award Number: DAMD17-99-1-9468

TITLE: Mitochondrial Apoptosis: A New Foundation for Combing Agents in Prostate Cancer Treatment

PRINCIPAL INVESTIGATOR: Charles E. Myers, M.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22906

REPORT DATE: March 2000

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2000	Annual (15 Feb 99 - 14 Feb 00)

4. TITLE AND SUBTITLE
Mitochondrial Apoptosis: A New Foundation for Combing Agents in Prostate Cancer Treatment

5. FUNDING NUMBERS
DAMD17-99-1-9468

6. AUTHOR(S)
Charles E. Myers, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, Virginia 22906

E-Mail: snuffy@virginia.edu

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. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Mitochondrial Apoptosis: A New Foundation for Combing Agents in Prostate Cancer Treatment. Charles E. Myers, M.D.

This grant sought to examine synergy between androgen withdrawal and drugs known to have activity against hormone-refractory prostate cancer. The hypothesis is that apoptosis induced by these various agents would converge on mitochondria enhancing tumor cell kill. During the first year, we were able to clearly show that none of the agents tested were synergistic or even additive with hormonal therapy. However, we did find promising synergy between HMG-Co reductase inhibitors or phenylbutyrate on one hand and the chemotherapy drugs, taxol, etoposide and suramin.

Our original hypothesis of synergy between androgen withdrawal and apoptosis induced by chemotherapy was predicted on the idea that all of these agents converged on mitochondria as the common pathway to apoptosis. We sought the explanation for the failure of this hypothesis. We found that prostate cancer cells contain large amounts of preformed FAS and FAS-L that are sequestered at distinct intracellular sites. In addition to mitochondrial apoptosis, rapid translocation of FAS or FAS-L to the cell surface represent a second independent pathway to cell death.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate Cancer	88
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

(EM Where copyrighted material is quoted, permission has been obtained to use such material.

(GM Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

(CM Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

5/26/00

Table of Contents

Introduction

This grant started with the observation that the response of prostate cancer to hormonal therapy was rarely complete. The goal of this grant was to find a drug or drugs that would act synergistically with androgen ablation to induce a complete response. The experimental approach was based on two observations: (1) one of the most prevalent forms of apoptosis involve release of cytochrome C from mitochondria, leading to activation of caspase 3 and onset of cell death; (2) normal and malignant prostate cells are one of the tissues richest in mitochondrial content. From these observations, we hypothesized that a promising strategy would be to seek drugs that enhanced the ability of androgen withdrawal to cause mitochondrial release of cytochrome C. To this end, we proposed to measure synergy between drug combinations using cell death, mitochondrial release of cytochrome C and caspase 3 activation as end points. The agents we selected for testing were drawn from the list of agents thought to have useful activity against prostate cancer.

Body

Task 1

Task 1 involved finding the most effective combination of androgen withdrawal with other apoptosis inducers. The cell line we chose for these experiments was LNCaP, a human cell line whose growth is stimulated by androgen, but that does not die when androgen is withdrawn. Thus, it is a model for human prostate cancer that incompletely responds to hormonal therapy.

Androgens are present in normal tissue culture media, largely associated with fetal calf serum. In order to induce androgen withdrawal, we added the antiandrogen, Casodex. Casodex alone at concentrations as high as 100 μM caused at maximum a 40% decline in cell number after five days (Appendix 1).

We then combined Casodex with each of the drugs listed in the proposal. None of the drugs interacted synergistically with Casodex (Appendix 1). In fact, in no case was the interaction even additive.

We then proceeded to examine the interaction between the other individual drugs, thinking that we might find synergy that might lead to a clinically useful combination that might be used independent of or before androgen withdrawal. Here we had considerable success and have discovered two nontoxic drugs, mevastatin and phenylbutyrate, that appear to markedly synergize with widely used chemotherapy drugs.

Mevastatin was used as an example of a large class of HMG CoA reductase inhibitors widely used to treat hypercholesterolemia. In previously published articles we had shown that these drugs induce apoptosis prostate cancer and brain tumor cells at concentrations nontoxic to most other mammalian cells [1]. Structure-activity studies showed that antitumor activity correlated with octanol/water partition coefficient with lovastatin, simvistatin and atorvastatin exhibiting the greatest potency. We followed this by a Phase I clinical trial in which we escalated the dose of lovastatin[2]. We hit dose-limiting toxicity at a dose of 4,000 grams per day. In this case, the toxicity was muscle weakness that correlated with depressed coenzyme Q10 levels and was reversed by oral administration of coenzyme Q10. Mevastatin markedly synergized etoposide, suramin, and taxol (Appendix 2). This finding is quite interesting in that the combination of etoposide and taxol is associated with response rates in excess of 50% in men with hormone-refractory prostate cancer. We think these findings justify further testing of the three-drug combination of etoposide, taxol and an HMG CoA reductase inhibitor.

Phenylbuytrate and phenylacetate are members of a new class of cancer drugs called aromatic fatty acids that act as nontoxic differentiation inducers[3]. We had played a lead role in the Phase I and Phase II testing of these drugs[4]. We recently reviewed the promise and problems associated with this drug class[5]. In addition to inducing tumor differentiation, this drug class can also induce apoptosis, particularly phenylbutyrate[6]. This drug proved to be synergistic with taxol (Appendix 2).

Why should HMG CoA reductase and phenyl-fatty acids share a synergistic relationship with taxol? All of these drugs are reported to block isoprenylation of ras family proteins, leading to apoptosis.

Why synergy was not found in the interaction between androgen withdrawal and various chemotherapy agents?

Our hypothesis was that mitochondrial leak of cytochrome c would link all of these agents. That concept proved to be false. In addition to mitochondrial apoptosis, the other major mechanism of cell death involves activation of the death receptors, including FAS and the receptors for tumor necrosis factor and trail. When it became clear that mitochondrial apoptosis failed to explain the response of prostate cancer, we examined the importance of death receptors in prostate cancer survival. What we discovered had not previously been described. It turns out that human prostate cancer cells contain enormous pools of both FAS and its ligand, FAS-L sequestered separately intracellularly. When we added an inhibitor of 5-lipoxygenase, MK886, both FAS and its ligand translocate to the cell surface within 30 minutes and initiate apoptosis that is complete within 8 hours (Appendix 3). We can find no other cell lineage in which both FAS and FAS-L are preformed and sequestered in this fashion.

This would be of little interest if this was limited to MK886, but a set of recently published observations suggest that a similar process is involved in apoptosis induced by androgen ablation. While not studied in prostate cancer, FAS/FAS-L appear to be involved in the death of other cell types after exposure to radiation or etoposide. In order to clarify the situation with regard to prostate cancer, we next studied the impact of taxol and etoposide on FAS and FAS-L in prostate cancer cell lines. We found that both agents caused translocation of FAS, but not FAS-L to the cell surface. This explains a previous publication that noted exposure to chemotherapy sensitized human prostate cancer to FAS-mediated cell killing[7].

Two survival pathways are now known to play a role in the survival of prostate cancer cells undergoing androgen withdrawal. One, bcl-2, was known at the time this proposal was submitted and task 2 proposed to test the ability of combinations to overcome this pathway. Bcl-2 appears to block cell death largely by preventing mitochondrial apoptosis. The second pathway involves signaling from the insulin receptor through PI3 kinase and Akt. PTEN is a protein that causes dephosphorylation of PI3 kinase and thus suppresses this survival pathway. This pathway appear to be of great importance because it controls both mitochondrial apoptosis and FAS receptor function[8]. Deletion of PTEN causes constitutive activation of this survival pathway and is a common finding in hormone-refractory prostate cancer[9]. Elevated blood levels of insulin-like growth factor 1, a ligand that activates this pathway has proved to be an important predictor of patient survival.

In the human prostate cancer cell line, LNCaP, PTEN is nonfunctional and this survival pathway is constitutively activated. MK886, which causes rapid translocation of FAS and FAS-L to the cell surface, very efficiently kills LNCaP, indicating that death mediated by simultaneous translocation of FAS and FAS-L by passes this important survival pathway. We have not yet tested the ability of this FAS-mediated mechanism of cell death to bypass bcl-2 as outlined in Task II.

<u>Recommendations for Further Work</u>

(1) Further examination of the synergy between HMG-CoA inhibitors and etoposide plus taxol with the goal of designing a clinical trial.

(2) Further examination of the synergy between phenylbutyrate and taxol as well as other chemotherapy agents with the goal of designing a clinical trial.

(3) Test for synergy between androgen withdrawal and agents that trigger rapid translocation of FAS/FAS-L to the cell surface.

Key Research Accomplishments

•HMG-CoA reductase inhibitor, mevastatin, interacts synergistically with etoposide, taxol and suramin.

•The orally active nontoxic differentiation inducer, phenylbutyrate, interacts synergistically with taxol.

•The 5-lipoxygenase inhibitor, MK886, induces cell death by a previously undescribed pathway involving simultaneous translocation of preformed FAS and FAS-L to the cell surface. This mode of cell death is independent of the PI3 kinase/Akt known to play a critical role in the development of hormone-resistant prostate cancer.

•Most chemotherapy drugs known to be active against prostate cancer are not synergistic or even additive with androgen withdrawal.

Reportable Outcomes

•The insights generated during the first year of this grant have led to the submission of a grant that proposes to systematically delete known survival pathways to see if better responses to hormonal therapy may be obtained. This proposal is included as a part of a Prostate Cancer SPORE grant submitted to NCI in February, 2000 (Appendix 4).

•This work has led to an abstract presented at the annual Australian meeting of Medical Oncologists and Radiation Therapists in Sydney in April that presented the synergy between taxol and HMG-CoA reductase inhibitors. A clinical trial is planned based on these results.

•A manuscript reporting the novel biology of FAS and FAS-L in prostate cancer is in preparation.

Conclusions:

1) Most available chemotherapy drugs are not synergistic or even additive with androgen withdrawal, suggesting relatively promise in this approach.

2) Synergy was observed between HMG CoA reductase inhibitors and etoposide, taxol and suramin. A clinical trial is planned testing taxol plus HMG CoA reductase inhibitors. Further testing of the three-drug combination of etoposide, taxol and HMG CoA reductase inhibitors should be done.

3) Synergy was observed between phenylbutyrate and taxol. Phenylbutyrate should be further tested for synergy with other drugs known to be active against prostate cancer.

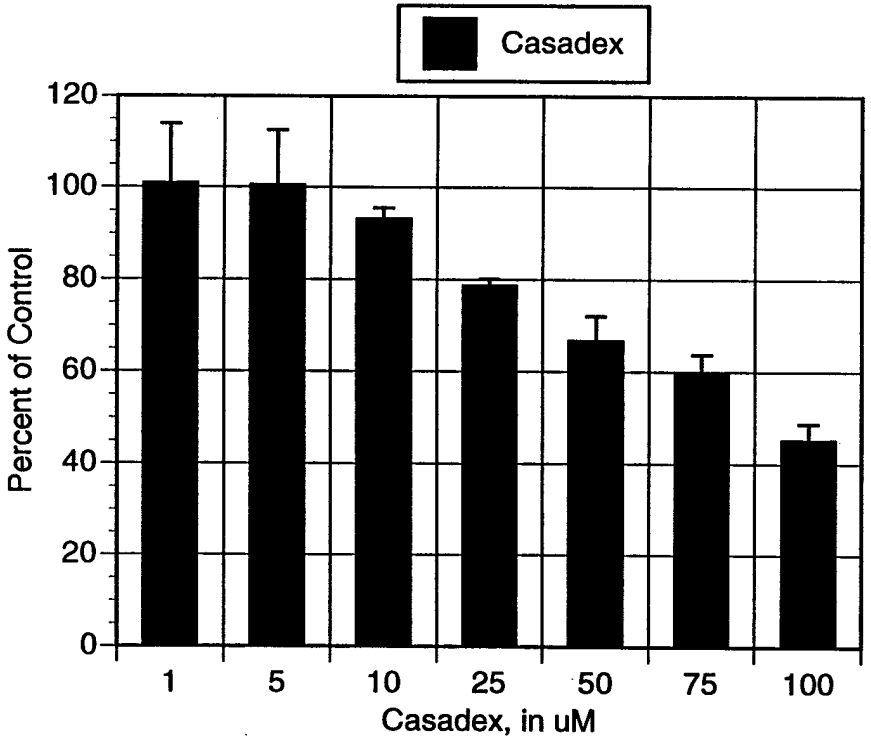
4) In addition to mitochondrial apoptosis, FAS/FAS-L appears to offer a promising therapeutic target.

Bibliography:

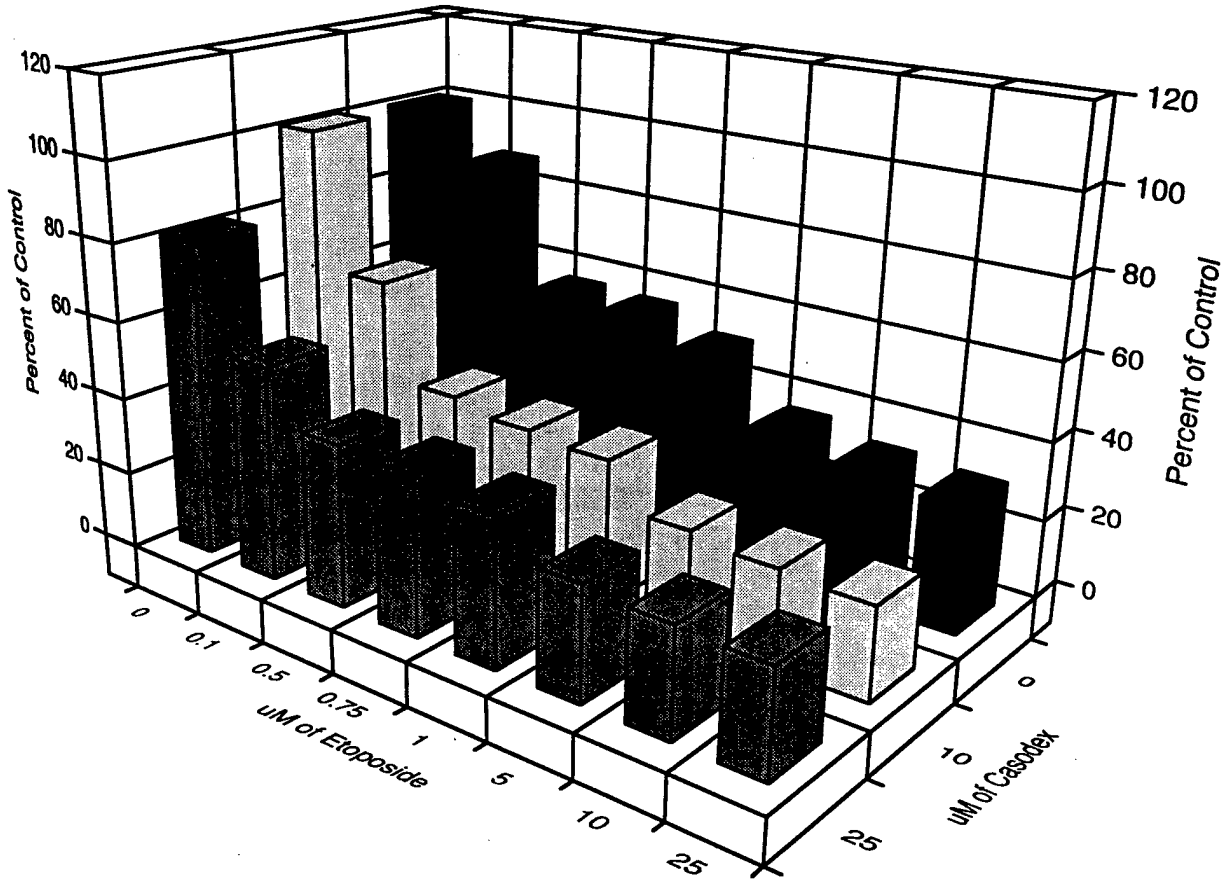
1. Borner, M.M., C.E. Myers, O. Sartor, Y. Sei, T. Toko, J.B. Trepel, and E. Schneider, *Drug-induced apoptosis is not necessarily dependent on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3.* Cancer Res, 1995. **55**(10): p. 2122-8.
2. Thibault, A., D. Samid, A.C. Tompkins, W.D. Figg, M.R. Cooper, R.J. Hohl, J. Trepel, B. Liang, N. Patronas, D.J. Venzon, E. Reed, and C.E. Myers, *Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer.* Clin Cancer Res, 1996. **2**(3): p. 483-91.
3. Samid, D., W.R. Hudgins, S. Shack, L. Liu, P. Prasanna, and C.E. Myers, *Phenylacetate and phenylbutyrate as novel, nontoxic differentiation inducers.* Adv Exp Med Biol, 1997: p. 501-5.
4. Piscitelli, S.C., A. Thibault, W.D. Figg, A. Tompkins, D. Headlee, R. Lieberman, D. Samid, and C.E. Myers, *Disposition of phenylbutyrate and its metabolites, phenylacetate and phenylacetylglutamine.* J Clin Pharmacol, 1995. **35**(4): p. 368-73.
5. Myers, C.E., *Differentiating agents and nontoxic therapies.* Urol Clin North Am, 1999. **26**(2): p. 341-51, ix.
6. Melchior, S.W., L.G. Brown, W.D. Figg, J.E. Quinn, R.A. Santucci, J. Brunner, J.W. Thuroff, P.H. Lange, and R.L. Vessella, *Effects of phenylbutyrate on proliferation and apoptosis in human prostate cancer cells in vitro and in vivo.* Int J Oncol, 1999. **14**(3): p. 501-8.
7. Uslu, R., N. Borsellino, P. Frost, H. Garban, C.P. Ng, Y. Mizutani, A. Belldegrun, and B. Bonavida, *Chemosensitization of human prostate carcinoma cell lines to anti-fas-mediated cytotoxicity and apoptosis.* Clin Cancer Res, 1997. **3**(6): p. 963-72.
8. Khwaja, A., *Akt is more than just a Bad kinase [news; comment].* Nature, 1999. **401**(6748): p. 33-4.
9. McMenamin, M.E., P. Soung, S. Perera, I. Kaplan, M. Loda, and W.R. Sellers, *Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage.* Cancer Res, 1999. **59**(17): p. 4291-6.

Appendix 1

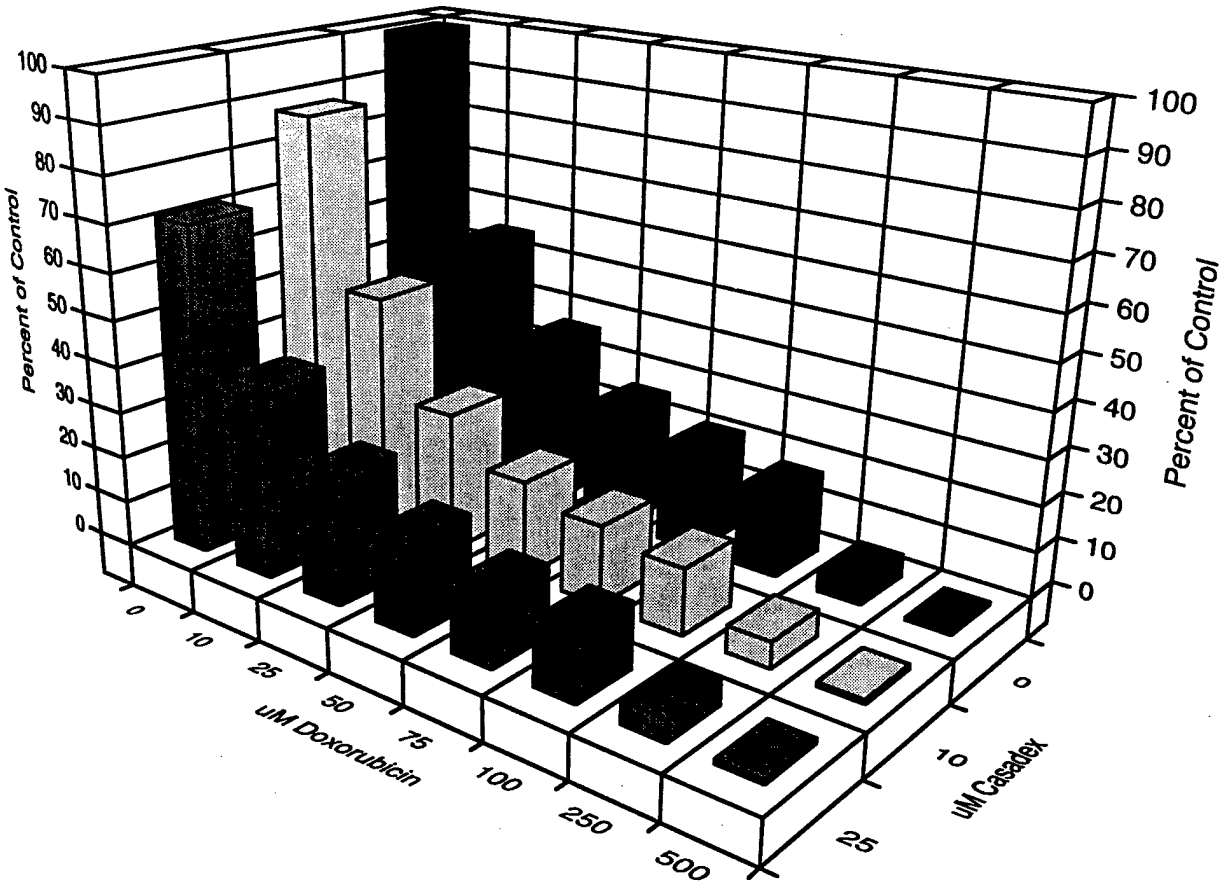
Casadex dose response curve for Army Grant Studies 12/27/99



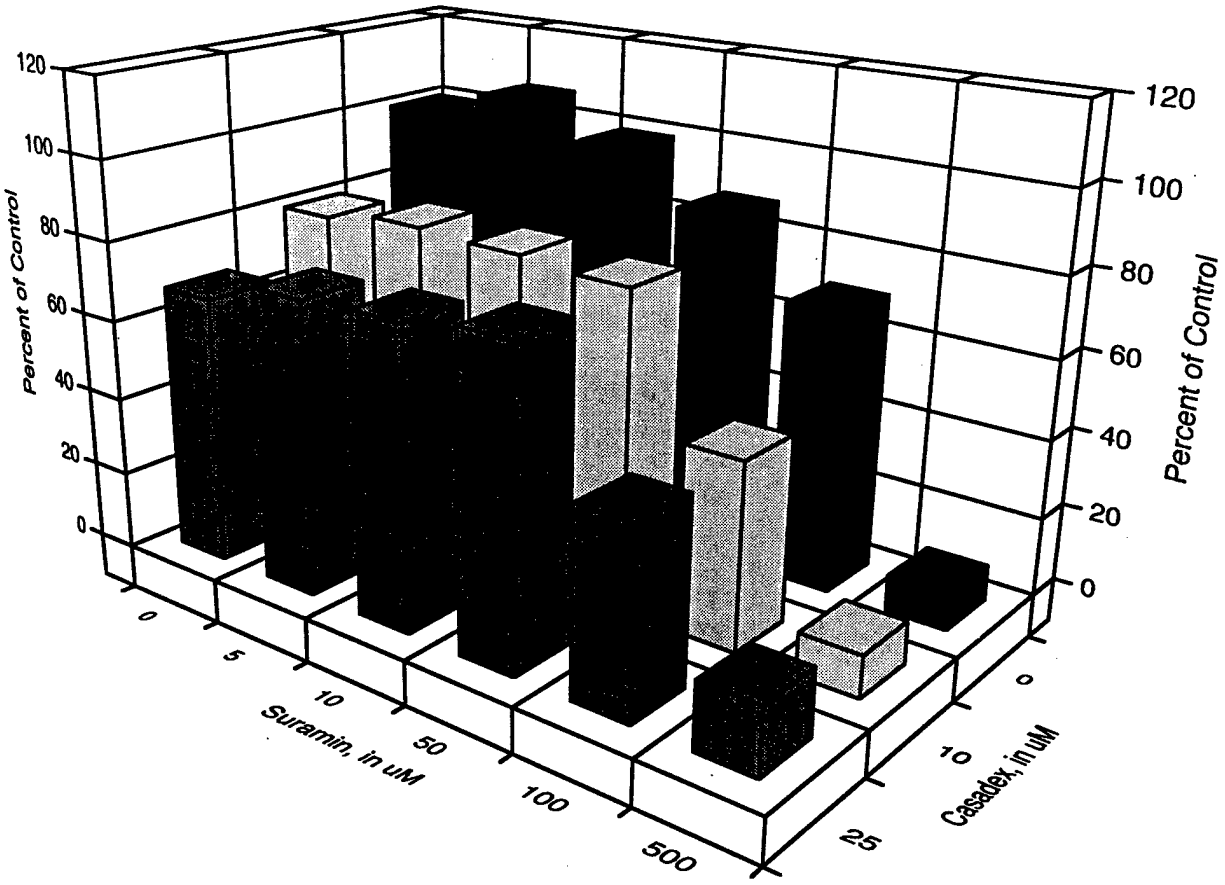
NCI LNCaP Against Etoposide
with varying Casodex in 10% FBS



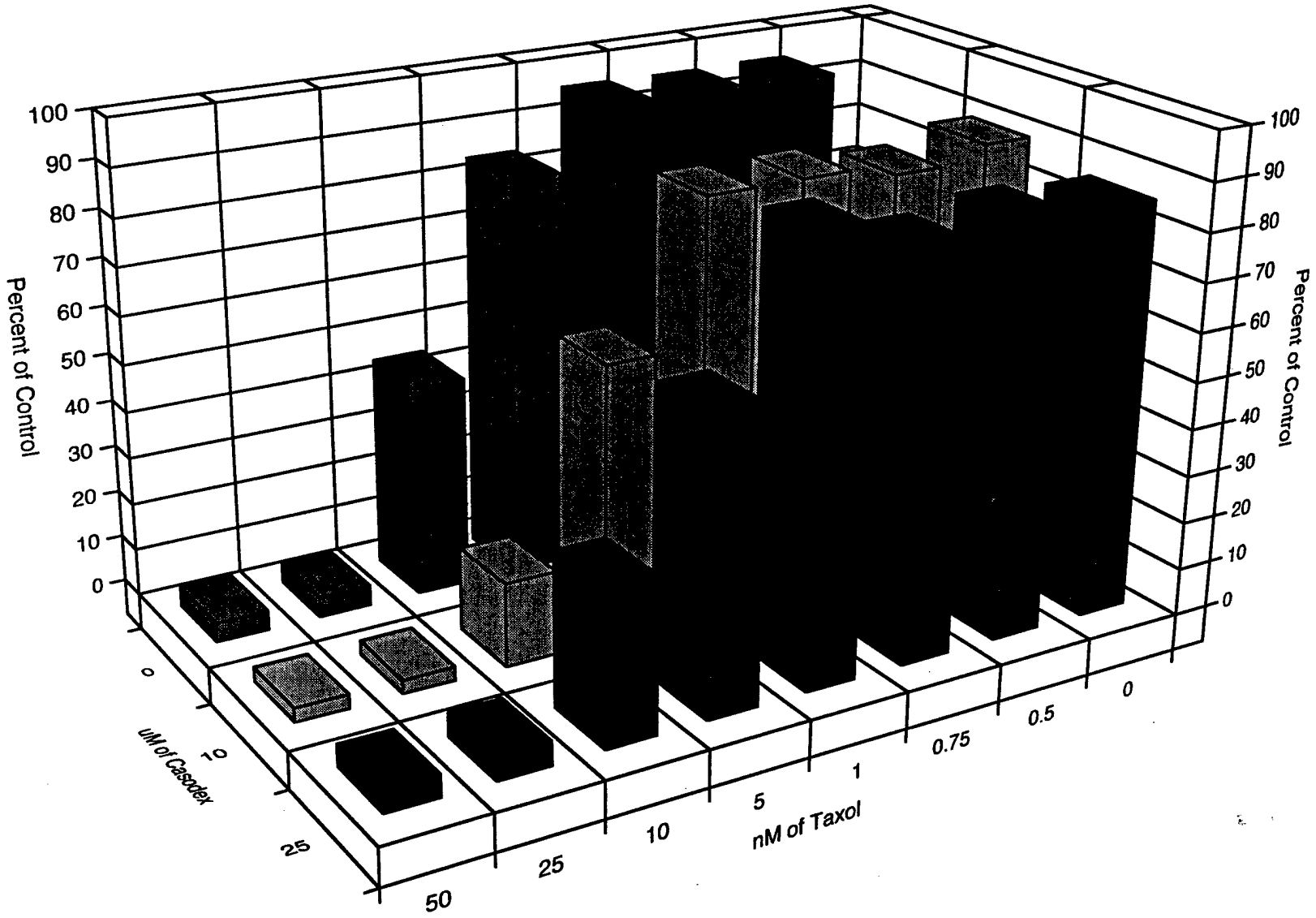
1/12/00 NCI LNCaP Against Doxorubicin
with varying amounts of Casadex in 10%FBS



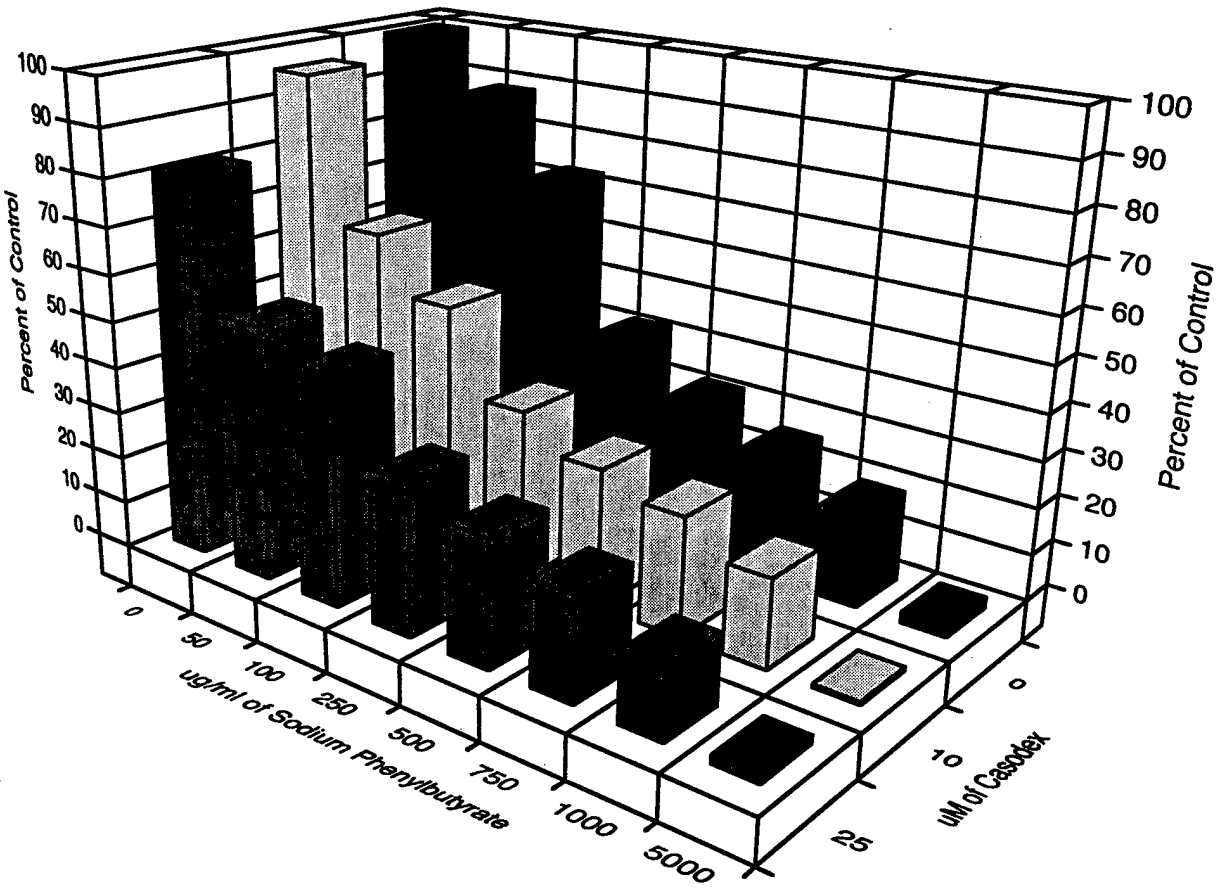
Suramin / Casadex Synergy Study
Army Grant Assay
12/27/99



1/12/00 NCI LNCaP Against Taxol
With Varying Casodex

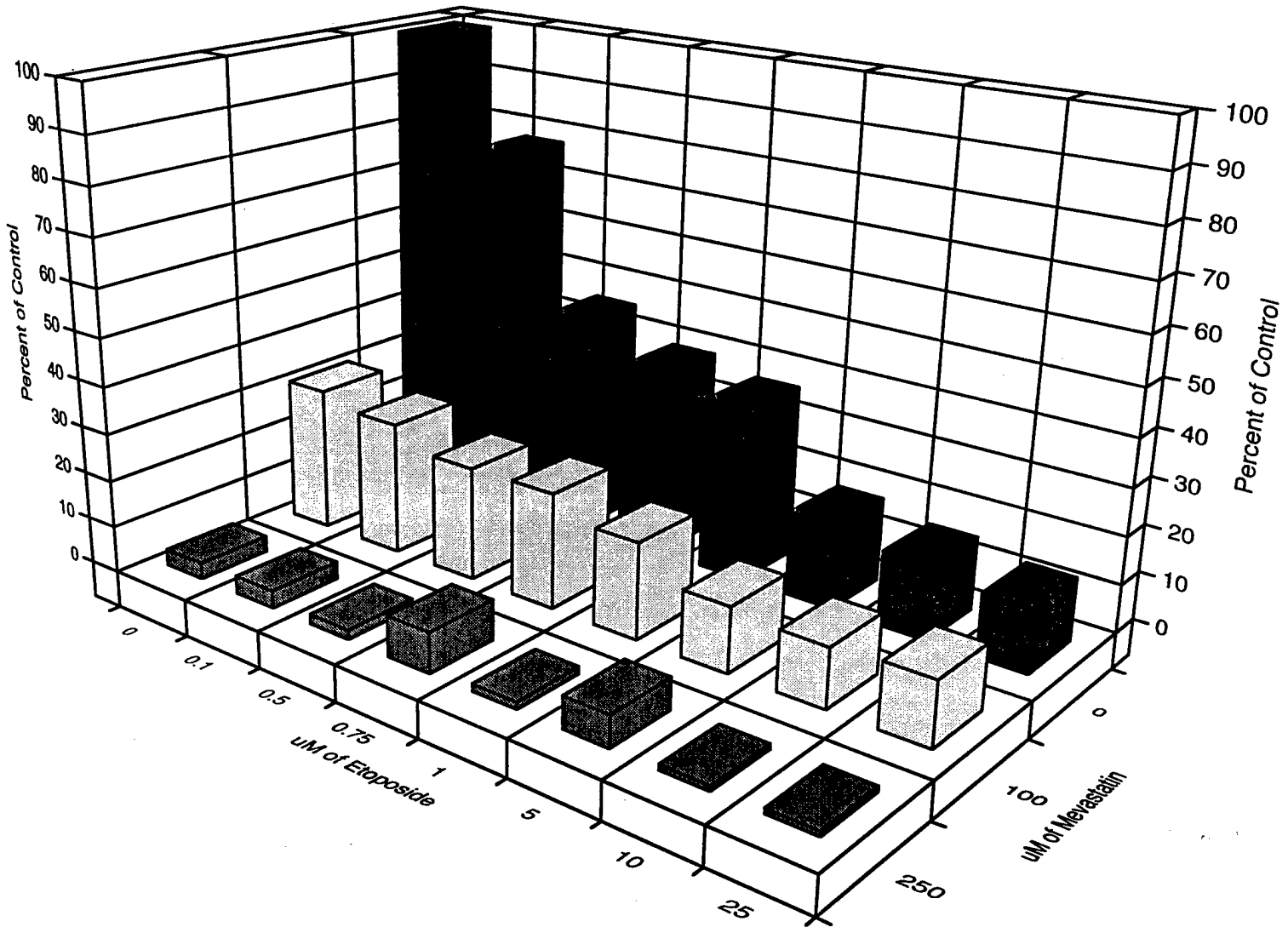


NCI LNCaP Against Sodium Phenylbutyrate
with varying amounts of Casodex in 10% FBS

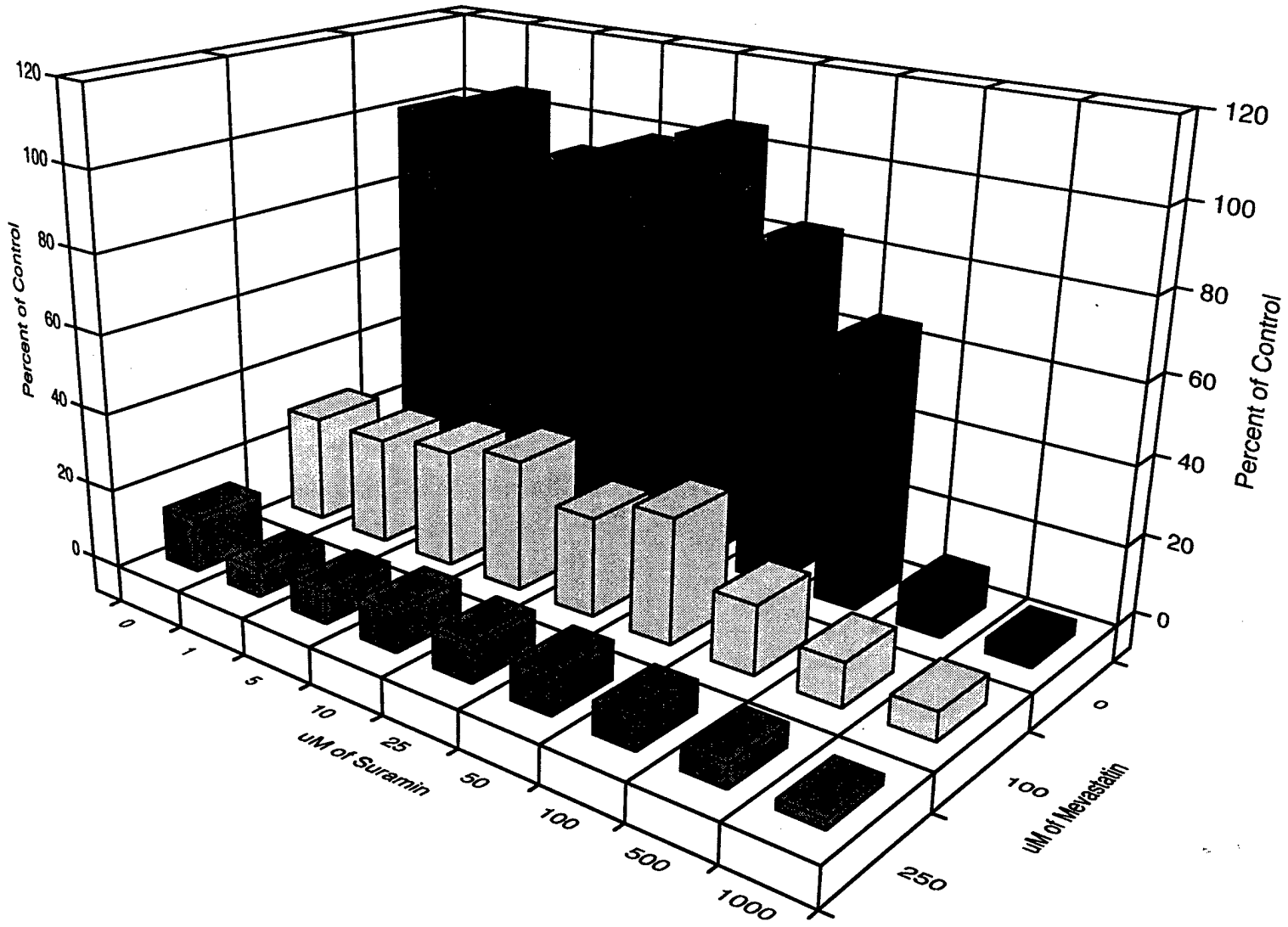


Appendix 2

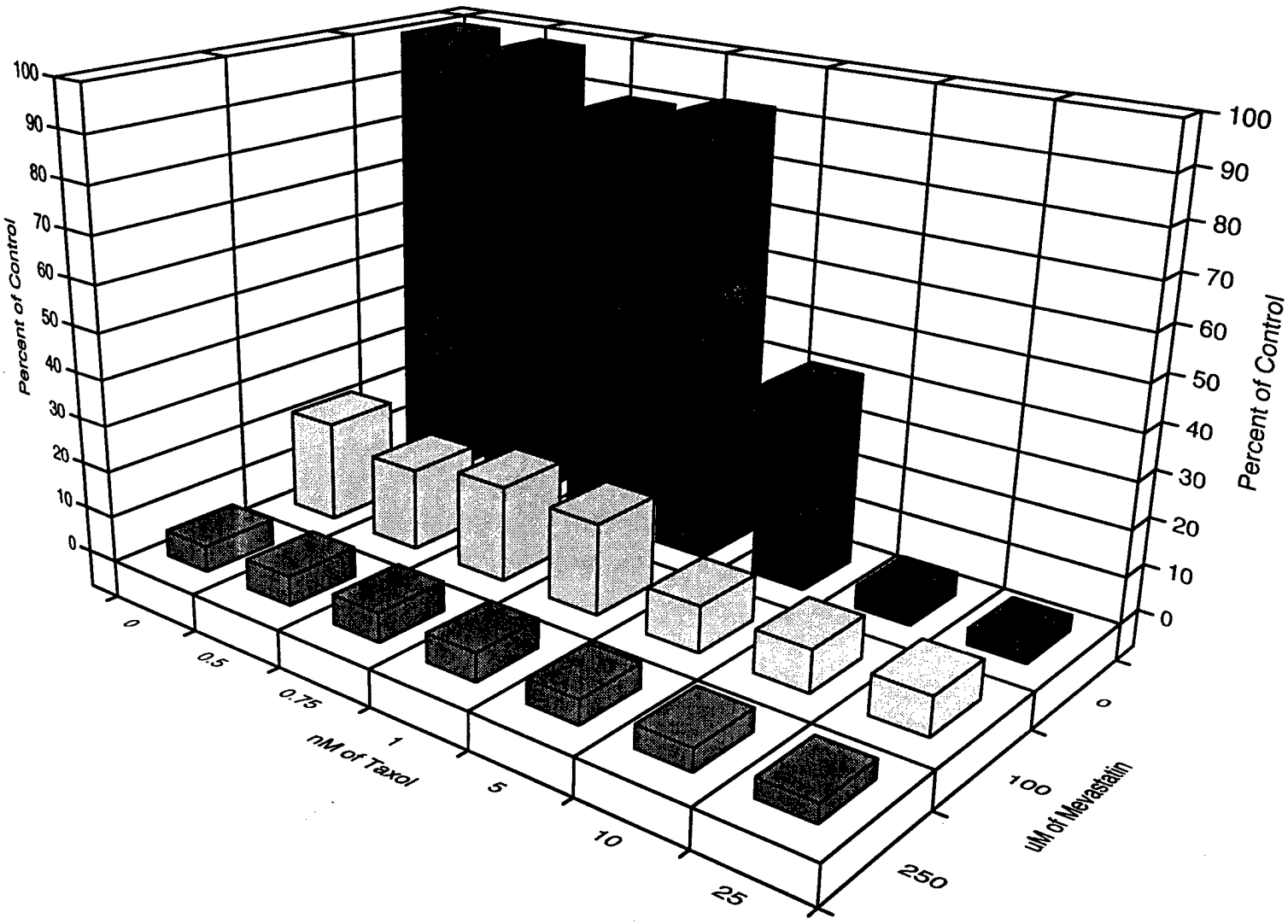
2/8/00 NCI LNCaP Against Etoposide
with varying amounts of Mevastatin in 10%FBS



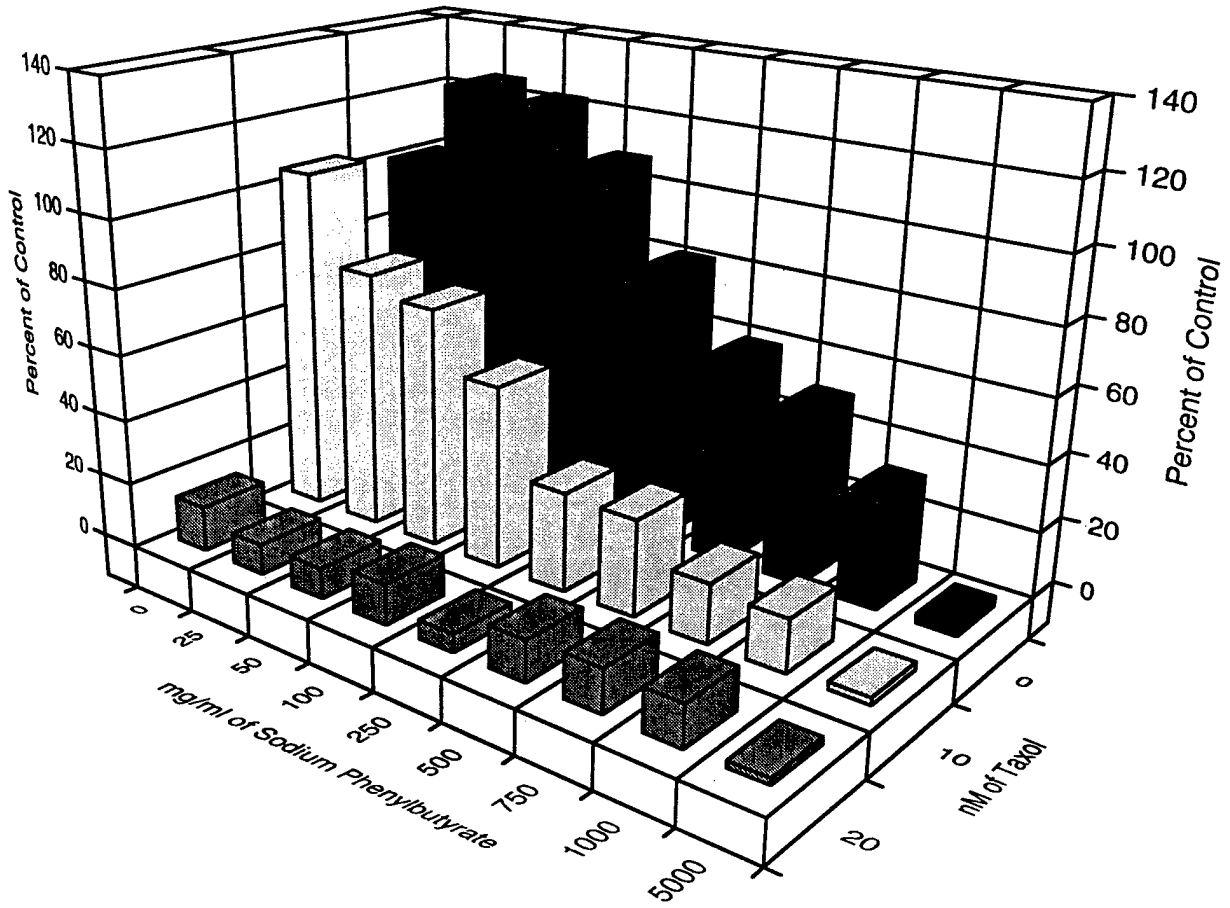
2/8/00 NCI LNCaP Against Suramin
with varying amounts of Mevastatin in 10% FBS



2/8/00 NCI LNCaP Against Taxol
With varying amounts of Mevastatin in 10%FBS



4/10/00 NCI LNCaP Against Sodium Phenylbutyrate
with varying amounts of Taxol



Appendix 3

CLINICAL RESEARCH 2

#266 AUTOLOGOUS FAS/FAS LIGAND-MEDIATED PROSTATE CANCER CELL SUICIDE TRIGGERED BY INHIBITION OF ARACHIDONATE 5-LIPOXYGENASE ACTIVITY. J Ghosh, and C E Myers, Jr., *Univ of Virginia Cancer Ctr, Charlottesville, VA*

Recently, we have reported that human prostate cancer cells undergo massive apoptotic death when the metabolism of arachidonic acid through 5-lipoxygenase is interrupted (Ghosh and Myers (1998) Proc. Natl. Acad. Sci. USA 95: 13482–13487). In our efforts to explore the molecular mechanisms underlying this rapid, synchronous cell death we found that the 5-lipoxygenase inhibition-induced prostate cancer cell death involves Fas death receptor-mediated signals, and occurs through the activation of CPP32 subfamily caspases. Prostate cancer cells treated with MK886, a specific inhibitor of arachidonate 5-lipoxygenase, showed a dramatic increase in the surface expression of both Fas death receptor and Fas ligand (FasL) within 30 minutes of treatment, which could be prevented by exogenous addition of 5(S)-HETE, a metabolic product of 5-lipoxygenase. Analysis of the death-inducing signaling complex (DISC) revealed that MK886 treatment increased the association of FADD (Fas-Associated Death Domain-containing protein) and caspase 8 with Fas receptor, as well as the enzymatic activity of caspase 8. Activation of caspase 8 was detected after 1–2 hours of MK886 treatment, whereas CPP32-like activity was observed after 2–3 hours which peaked at 6–8 hours post treatment. Finally, blocking Fas death receptor-mediated signals with neutralizing anti-Fas or anti-FasL antibodies substantially reduced MK886-treated prostate cancer cell death. These findings suggest that in human prostate cancer cells, 5-lipoxygenase metabolites of arachidonic acid regulate Fas/FasL-mediated signals to programmed cell death.

#267 *TP53* STATUS AND SURVIVAL OF PATIENTS WITH CLINICALLY LOCALIZED PROSTATE CANCER TREATED WITHOUT INTENT TO CURE. Jan Alsner, Michael Borre, and Jens Overgaard, *Aarhus Univ Hosp, Aarhus, Denmark*

OBJECTIVES: In order to identify whether *TP53* status is a prognostic marker for prostate cancer, *TP53* mutation is correlated with disease-specific survival and overall survival in patients with clinically localized prostate cancer treated with no intent to cure. METHODS: The patients included are part of a complete prostate cancer population consisting of 719 persons diagnosed between 1979-1983. Following exclusion of patients diagnosed postmortem, not subjected to palliative prostate surgery at diagnosis, with incomplete data registration, or archival tumor tissue not available, the study population was reduced to 221 patients. Of these patients, 125 were diagnosed with clinically localized (T1-2, Nx, M0) prostate cancer. *TP53* analysis was not performed in 39 cases, leaving 86 patients in this study. DNA was extracted from archival formalin fixed, paraffin embedded tumor tissue obtained at diagnosis. Gene mutations (in exons 5-9) were identified using Denaturing Gradient Gel Electrophoresis (DGGE) as the initial scanning procedure and characterized by sequencing. RESULTS: *TP53* mutations were found in 26 patients (30%). From these patients, one-fourth of the samples contained more than one mutation. No correlation was found between the presence of *TP53* mutation(s) and disease-specific or overall survival. Neither was there any correlation between specific types of mutations (missense mutations affecting DNA-contact or Zn-binding regions) and disease-specific or overall survival. CONCLUSIONS: In patients with clinically localized prostate cancer treated without intent to cure, *TP53* mutation does not appear to be a prognostic marker for disease-specific or overall survival.

#268 P53 FUNCTIONAL STATUS AND APOPTOTIC REGULATION IN BENIGN PROSTATIC HYPERPLASIA (BPH). Philip C Mack, Regina Gandour-Edwards, William S Holland, Salvador N Toscano, Arline D Deitch, Ralph W deVere White, and Paul H Gumerlock, *UC Davis Cancer Ctr, Sacramento, CA*

BPH is a slowly progressing prostatic enlargement with heterogeneous morphology. Disruption of apoptotic pathways has been suggested as an important regulatory mechanism in this disease. To investigate this hypothesis, BPH specimens from 47 patients with no evidence of prostatic carcinoma were obtained by transurethral prostatectomy (TURP) at the University of California, Davis and analyzed by immunohistochemistry (IHC). Of these, 7 (15%) had greater than 10% glandular secretory cells positive for Bcl2, while 21 (45%) similarly showed accumulated p53, suggestive of abnormalities in these genes. Selected samples were further evaluated for Bax and p27 expression. Particularly striking was the decreased expression of Bax within epithelial nodules relative to other glandular elements. Additionally, p27 expression was decreased in some hyperplastic areas and nodules. Overall, all hyperplastic nodules showed one or more IHC abnormalities. Laser Capture Microdissection (LCM) was used to isolate p53-positive secretory cells. Single-stranded conformational polymorphism (SSCP) analysis was used to identify alterations in the p53 gene. Of five samples studied, two showed abnormalities in exon 5 and one in exon 7, confirmed by DNA sequencing to be missense mutations (exon 5 and 7) and a nonsense mutation (exon 5). The two samples with exon 5 mutations were used in an adaptation of the yeast assay that scores p53 transactivational function in archival specimens. In both cases, the p53 alleles showed a loss of function. IHC-negative areas from the same slides consistently yielded background levels of red colonies. These data suggest that disruption of apoptotic pathways are common in BPH. [Supported by NIA (AG15404) and NCI (CA77662)]

#269 BLOCKADE OF CONSTITUTIVELY ACTIVATED STAT3 SIGNALING PATHWAY SUPPRESSES GROWTH OF PROSTATE CANCER CELLS. Allen C Gao, Zuyao Ni, and Wei Lou, *Univ of Pittsburgh, Pittsburgh, PA*

Overexpression of interleukin-6 (IL-6), a down-stream target of GBX2 homeobox gene, has been linked to the progression of prostate cancer. The Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) signaling pathway transmits IL-6 mediated signals from cell surface receptors to the target genes in the nucleus and is critical in mediating cellular growth and differentiation. We demonstrate that cells derived from both rat and human prostate cancers have constitutively activated Stat3 with Stat3 activation being correlated with malignant potential. Blockade of the activated Stat3 by ectopic expression of a dominant-negative Stat3 in human prostate cancer cells significantly suppresses their growth in vitro and tumorigenicity in vivo. Furthermore, the JAK kinase inhibitor, tyrphostin AG490, inhibited the constitutive activation of Stat3 and suppressed the growth of human prostate cancer cells. These results indicate that activation of the Stat3 signaling is essential in the progression of prostate cancer cells and suggest that targeting Stat3 signaling may yield a potential therapeutic intervention for prostate cancer.

#270 STAT3 ACTIVATION IN PROSTATE CARCINOMA. Linda B Mora, J Seigne, J I Diaz, R Garcia, T Bowman, A Cantor, J Pow-Sang, and R Jove, *H Lee Moffitt Cancer Ctr & Res Inst, Tampa, Fl*

Signal Transducers and Activators of Transcription (STATs) were originally identified as key components of signaling pathways involved in mediating responses to cytokines. STAT proteins may have important roles in normal cellular growth control and malignant transformation associated with tyrosine kinase signaling pathways. Constitutive activation of STAT3 by IL-6 plays an essential role in the pathogenesis of multiple myeloma by preventing apoptosis. As IL-6 is reported to be a growth modulator in prostate cancer cell lines, we examined IL-6 mediated STAT activation in the human prostate cancer cell line LNCaP. We assayed constitutive STAT3 activation in 2 additional prostate cancer cell lines (Du145, Pc-3) and 11 primary tumors (with matched normal prostate) obtained at radical prostatectomy. STAT DNA-binding activity was assayed by an electrophoretic mobility shift assay with appropriate antibodies to detect the activated STAT3. Analysis of STAT DNA-binding activity in human prostate cancer cell lines showed constitutive activation of STAT3 in all cell lines examined. Substantially lower levels of STAT3 activation are seen in LNCaP; however, a significant increase in STAT3 DNA-binding occurred following stimulation with IL-6. Furthermore, high levels of constitutive STAT3 activation were detected in 8 of 11 (73%) of the primary prostate tumors compared to the matched normal prostate tissues. These findings demonstrate that constitutive activation of STATs occurs frequently in prostate cancer cell lines and primary prostate tumors. Our studies suggest that aberrant STAT activation may be involved in the pathogenesis of prostate cancer.

#271 THE INFLUENCE OF NITRIC OXIDE AND IT'S CORRELATION TO THE EXPRESSION OF NITRIC OXIDE SYNTHASE IN PROSTATE CANCER. R Bhatti, P Ray, J Gadarowski, E Ali, N Bana, A Zeba, C Lash, S Stokes, and K Basheeruddin, *Cook County Hosp, Chicago, IL*

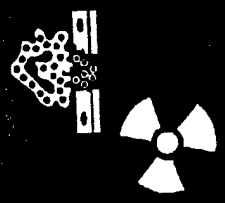
The pathophysiologic role of nitric oxide (NO) in prostate cancer (Pca) is unclear. We measured NO and Nitric Oxide Synthase (NOS) in Pca and in benign prostatic hypertrophy (BPH), in sera from patients in various stages of Pca, and in tumor cells and tumor fluids from R-3327 tumors to evaluate the effect of NO & NOS on the behavior and metastatic potential of Pca. NO was measured by a correlate assay in which nitrate is converted to nitrite by the enzyme nitrate reductase followed by the colorimetric detection of nitrite as a colored dye product of the Griess reaction. NOS activity was analyzed by using [3H] arginine as a substrate in the presence of 3M tetrahydro biopterin, 1μ FAD & FMN, and 1.25 mM of $CaCl_2$. The formation of [3H] citrulline was measured and expressed as cpm/mg protein/hour. Our data show that NO was significantly higher in Pca as compared to BPH. Similarly NO level increased significantly in metastasizing AT3 tumor as compared to non-metastasizing G-line. NOS expression in AT3 tumor was parallel to NO level and was much higher in AT3 than G-line tumor, indicating a direct correlation between NO & NOS and its influence on the metastatic potential. In sera, NO level dropped gradually with the advancing Pca with lowest level in stage D_2. Most of these patients were on Lupron/Flutamide therapy. Whether such drop in NO level was the effect of this therapy, needs further investigation. NO level was also higher in tumor fluids from AT3 as compared to G-line tumors suggesting a possible influence of NO on the micro environment of the tumor and the aggressive behavior of tumor cells.

#272 COX-2 EXPRESSION IN PROSTATE CANCER CELL LINES. Shan Zha, Jurgita Sauvageot, Charles Ewing, and William Isaacs, *Johns Hopkins Univ Sch of Medicine, Baltimore, MD*

Linkage analysis of prostate cancer families has identified chromosome 1q24–q25 as potentially harboring a prostate cancer susceptibility gene (*HPC1*). Cyclooxygenase-2 (COX-2), which resides in this region, has been reported to have high expression in a variety of human cancers. However, its role in prostate carcinogenesis is still unclear. We evaluated expression of COX-2 protein by immunohistochemistry (IHC) of tissue samples and western blotting of cell lines. IHC analysis of prostate tissue samples does not show consistent difference of COX-2 expression between prostate cancer and normal prostate in general, or

EICOSANOIDS & OTHER BIOACTIVE LIPIDS IN CANCER, INFLAMMATION & RELATED DISEASES

6th International Conference
THE WESTIN COPLEY PLACE
Boston, Mass. - September 12-15, 1999



FINAL PROGRAM AND ABSTRACT BOOK

84

CENTRAL ROLE OF ARACHIDONATE 5-LIPOXYGENASE IN THE REGULATION OF CELL GROWTH AND APOPTOSIS IN HUMAN PROSTATE CANCER CELLS

Jagadananda Ghosh and Charles E. Myers

University of Virginia Cancer Center
Charlottesville, Virginia 22908

Dietary fat and the risk of prostate cancer: Prostate cancer has emerged as the most frequently diagnosed malignancy among men in the United States, taking thousands of lives every year (1). It is a multistep disease. In the United States, while there are more than 15 million men with localized prostate cancer, only 40-50,000 men will develop malignancy and die in a given year. Thus the development of the metastatic phenotype is an important rate limiting step in the natural history of prostate cancer. Through the incidence of the latent form of the diseases the same worldwide, there is significant geographic variation in the diagnosis of clinically evidence prostate cancer and mortality (2-4) suggesting involvement of environmental factors in this process. Moreover, people who migrate from low-incidence countries to high-incidence countries show increased risk of invasive prostate cancer (5-8) indicating a positive role for lifestyle factors, such as diet and nutrition.

Among the dietary factors, high intake of fat is frequently found to be associated with the diet in affluent nations, like the United States and Western Europe, where prostate cancer is also more common. Moreover, a number of epidemiological studies as well as experiments with animal models suggest a link between high dietary fat consumption and cancer of the prostate (9-14). Despite these reports, knowledge about the factors in fat associated with the risk of prostate cancer, such as high calories, a particular fat source or component fatty acid, is very limited. Based on this background information, our new project is focused on analysis of the effects of dietary fatty acids on the malignant behavior of human prostate cancer cells.

Androgen is a potent growth stimulator for prostate cancer cells. Fatty acids, e.g. arachidonic acid, stimulate production of androgen by the Leydig cells in testis and might have an indirect effect on the growth of cells of prostatic origin. Recent reports suggest a role for arachidonic acid, an omega-6 poly-unsaturated fatty acid, in the proliferation of prostate cancer cells (15-17). Moreover, we recently reported that arachidonic acid is a potent mitogen for both the androgen sensitive and androgen resistant human prostate cancer cells *in vitro* (18). Arachidonic acid also strongly stimulates *in vitro* invasion of human prostate cancer cells. Therefore, high intake of dietary fat rich in arachidonic acid might have a profound impact on the clinical progression of prostate cancer.

Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury, 4
Edited by Honn *et al.*, Kluwer Academic / Plenum Publishers, New York, 1999.

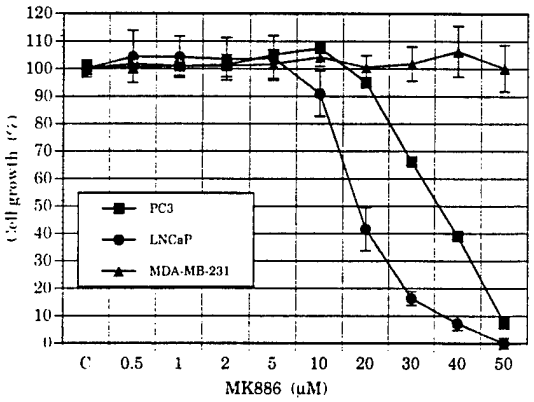


Figure 1. Inhibition of prostate cancer cell growth by 5-lipoxygenase specific inhibitor MK886. Cells (3000 per well) were plated overnight in RPMI medium supplemented with 10% FBS in 96 well tissue culture plates. On day-2, cells were treated with varying doses of MK886 and the plates were further incubated for 72 hours. Control cells were treated with the plating medium containing 0.02% DMSO. At the end of incubation period, cell growth was measured by MTS/PMS Cell Titer Assay (18). The results are presented as the mean ± standard error (n=8).

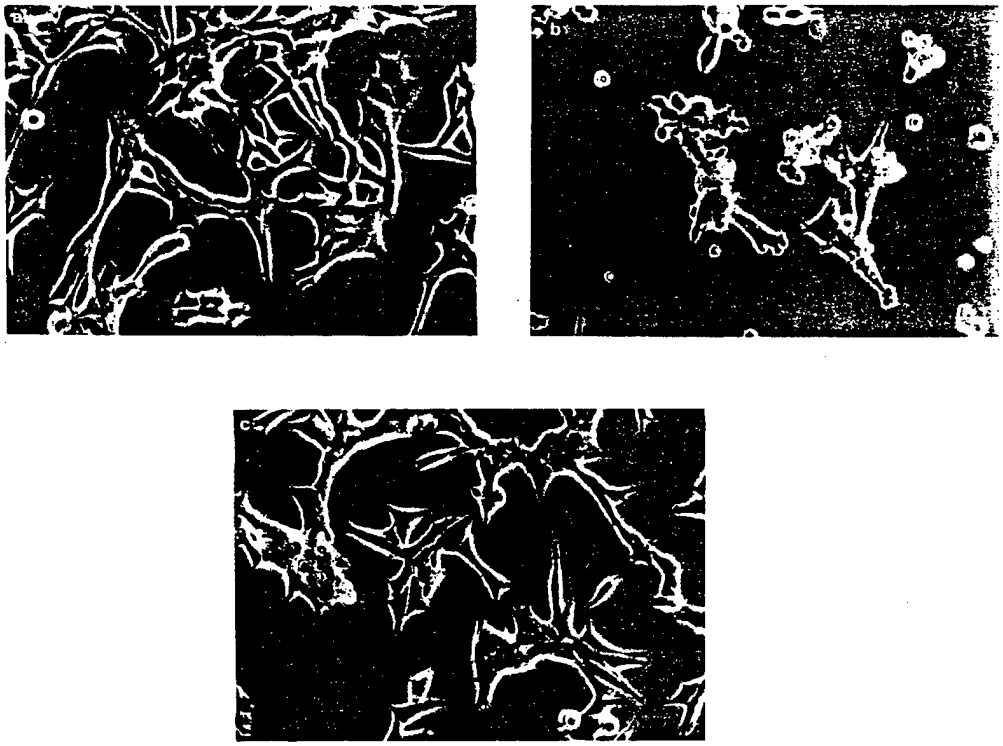


Figure 2. Photomicrographs showing morphology of LNCaP prostate cancer cells upon treatment with MK886. Cells (3 x 10^5) were plated in 60 mm diameter plates (Falcon) in RPMI medium supplemented with 10% FBS and grown for 48 hours. The old medium was then replaced with 2 ml fresh serum-free RPMI medium and the cells were treated with 10 μM MK886 for 6 hours with or without the addition of 5-lipoxygenase metabolite, 5-oxoETE (500 nM). Control cells were treated with serum-free medium containing 0.02% DMSO. Photographs were taken with a Zeiss inverted microscope at 20 X. (A) Control; (B) MK886 only; (C) MK886 plus 5-oxoETE.

Arachidonic acid metabolism, and prostate cancer cell growth an survival: Arachidonic acid and its precursor, linoleic acid, are essential fatty acids which we should obtain from dietary sources. Western style foods, particular rich in red meat and dairy products, are good source of these fatty acids. How these fatty acids modulate the malignant behavior of prostate cancer cells is an open question. After seeing the stimulatory effect of arachidonic acid on human prostate cancer cell growth, we were interested in the molecular mechanism involved in this process. Arachidonic acid can directly modulate the activity of a number of cellular proteins, like protein C and ras-GAP, known to have positive effects on malignant transformation (19-21). In addition to that, arachidonic acid can be metabolized to a wide range of eicosanoids (22, 23) which modulate diverse physiological and pathological functions including growth and invasion of tumor cells and suppression of immune surveillance (24, 25).

Using selective inhibitors of the various pathways of arachidonic acid metabolism, we found that metabolic conversion of arachidonic acid by 5-lipoxygenase is a critical requirement for its growth stimulation of prostate cancer cells. Inhibition of other major pathways of arachidonic acid metabolism (e.g. cyclooxygease, cytochrone P450, 12-lipoxygenase, etc.) did not show any appreciable effect. The hydroxydicosatetraenoid metabolites of 5-lipoxygenase (5-HETE, 5-HETE lactone and 5-oxoETE) exert mitogenic effects on prostate cancer cells and reverse the inhibitory effect of 5-lipoxygenase blockade. On the other hand, leukotrienes (e.g. LTB4, LTC4, etc.), another class of 5-lipoxygenase metabolites, are ineffective. These observations suggest that production of the 5-HETE series of eicosanoids is essential for arachidonic acid-stimulated growth of human prostate cancer cells. This proved to be the case: by specific radioimmunoassay measurement we demonstrated that prostate cancer cells treated with arachidonic acid produce 5-HETE and release this eicosanoid into the medium.

In other cell types, metabolism of arachidonic acid through cyclooxygenase and lipoxygenase pathways shows a strong correlation with growth factor-stimulated cell proliferation (26,27). Both EGF and androgen are well known mitogens for prostate cancer cells. Interestingly, inhibition of arachidonic 5-lipoxygenase blocks the mitogenic activity of these agents. Moreover, MK886, a specific inhibitor of 5-lipoxygenase activity, is a strong inhibitor of serum-stimulated growth of both androgen-responsive (LNCaP) and non-responsive (PC3) human prostate cancer cells, indicating an essential role for this pathway in the regulation of growth by these cells (Figure 1). Under the same experimental conditions, a hormone independent human breast cancer cell line, MDA-MD-231, is completely refractory to this inhibition.

Apoptosis, or programmed cell death, is a genetically based suicide mechanism preserved through evolutionary ages (28). It is the dominant mechanism of cell death in multicellular species. In prostate cancer cells, apoptosis is induced with treatment by drugs that bind with DNA or the cytoskeleton. Eicosanoids are now well known both as inducers and blockers of apoptotic cell death. The effect of a particular eicosanoid is cell type specific. Prostaglandin E_2 (PGE_2) is central to the apoptosis needed for egg release du ring ovulation (29), whereas it protects CD4+/CD8+ cells from activation-induced apoptotic cell death (30). Moreover, TNF and FAS-mediated cell deaths are associated with activation of phospholipase A2 and formation of lipoxygenase metabolites (31,32). Our previous observation of the dramatic inhibition of prostate cancer cell growth by selective inhibition of 5-lipoxygenase led us to explore whether operation of the 5-lipoxygenase pathway has any role in the survival of human prostate cancer cells.

We observed that inhibition of 5-lipoxygenase by MK886 induces a massive alteration of membrane morphology in prostate cancer cells, forming numerous blebs within hours of treatment. Inhibitors of other well characterized pathways of arachidonic acid metabolism (cyclooxygenase, cytochrone P450 or 12-lipoxygenase) did not cause any appreciable change in membrane morphology. Exogenous addition of 5-oxoETE, a metabolite of arachidonate 5-lipoxygenase, protects these cells from the severe membrane

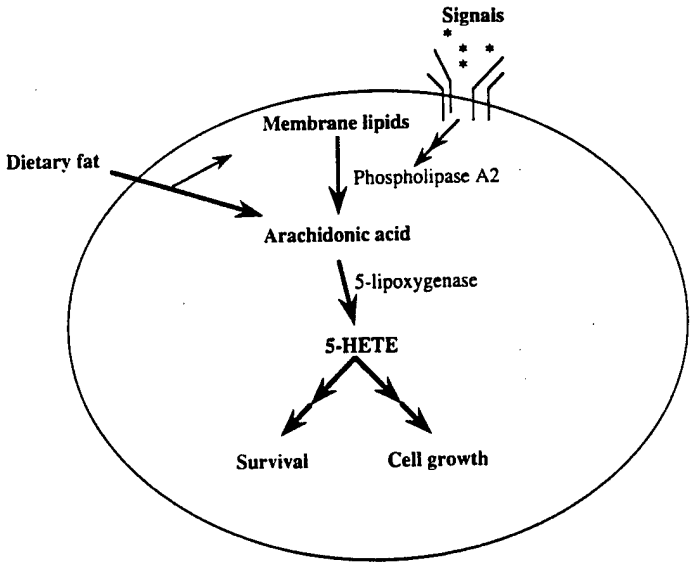


Figure 3. A model illustrating regulation of prostate cancer cell growth and survival by arachidonic acid.

changes induced by inhibition of 5-lipoxygenase, indicating a critical role for this pathway in the survival of prostate cancer cells (Figure 2). The extensive formation of membrane blebs suggest that it might be a case of apoptotic cell death. Later, we confirmed that the extensive formation of membrane blebs is associated with other standard features of apoptosis, e.g., mitochondrial permeability transition, externalization of phosphatidylserine and degradation of DNA to nucleosomal subunits.

The rapid induction of prostate cancer cell death upon inhibition of 5-lipoxygenase and its prevention by 5-HETE series of eicosanoids mean that these cells are constitutively making these metabolites. By direct immunoassay measurement, we demonstrated that both androgen sensitive (LNCaP) and androgen resistant (PC3) human prostate cancer cells consititutely produce 5-HETE in serum-free medium without any externally added stimulus. Moreover, this production of 5-HETE is dramatically increased upon treatment with exogenous arachidonic acid, indicating that prostate cancer cells can metabolize both endogenous and exogenous pools of arachidonic acid to produce a critical 5-lipoxygenase metabolite, 5-HETE.

Our experimental observations reveal that a continuous supply of 5-HETE is an absolute requirement for the survival and regulation of growth of both hormone responsive and non-responsive human prostate cancer cells. Based on our recent findings we want to propose a model showing the regulation of prostate cancer cell growth and survival by arachidonic acid metabolism (Figure 3).

ACKNOWLEDGMENTS

We express our sincere gratitude to the Lynch and Reynolds Foundation for their financial support. This work was also supported in part by the CapCure Foundation and an NCI Developmental Grant in Prostate Cancer, R21-CA 69848.

REFERENCES

1. Parker, S.L., Tong, T., Bolden, S., and Wingo, P.A. Cancer Statistics, 1997. *CA Cancer J. Clin.* 47:5-27 (1997).
2. Jackson, M.A., Ahluwalia, B.S., Herson, J., et al. Characterization of prostatic carcinoma among blacks: a continuation report. *Cancer Treat. Rep.* 61:167-172 (1977).
3. Brelow, N.E., Chan, C.W., Dhom, G., et al. Latent carcinoma of prostate at autopsy in seven areas. *Int. J. Cancer* 20:680-688 (1977).
4. Yatani, R., Chigusa, I., Akazaki, K., Stemmerman, G.N., Welsh, R.A., and Correa P. Geographic pathology of latent prostatic cancer. *Int. J. Çancer* 29:611-616 (1982).
5. Haenszel, W., and Kurihara, M. Studies of Japanese migrants. I. Mortality from cancer and other diseases among Japanese in the United States. *J. Nat. Cancer Inst.* 40:43-68 (1968).
6. Shimizu, H., Ross, R.K., Bernstein, L., Yatani, R., Henderson, B.E. and Mack, T.M. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br. J. Cancer* 63:963-966 (1991).
7. Muir, C.S., Nectoux, J., and Straszewski, J. The epidemiology of prostatic cancer: geographical disribution and time-trends. *Acta Oncol.* 30:133-140 (1991).
8. Yu, H., Harris, R.E., Gao, Y.T., Gao, R., and Wynder, E.L. Comparative epidemiology of cacers of the colon, rectum, prostate and breast in Shanghai, China versus the United States. *Int. J. Epidemiol.* 20:76-81 (1991).
9. Whittemore, A.S., Kolonel, L.N., Wu, A.H., John, E.M. Gallagher, R.P., Howe, G.W., Burch, J.D., Hankin, J., Dreon, D.M., West, D.W., Tehm C., and Paffenbarger, R.S. Prostate cancer in relation to diet, physical activity and body size, in blacks, white and asians in the U.S. and Canada. *J. Natl. Cancer Inst.* 87:652-661 (1995).
10. Mettlin, C., Selenskas, s., Natarajan, N., and Huben, R. Beta-carotene and animal fats and their relationship to prostate cancer risk. *Cancer* 64:605-612 (1995).
11. Giovannucci, E., Rimm, E.B., Colditz, G.A., Stampfer, M.J., Ascherio, A., Chute, C.C., and Wilett, W.C. A prospective study of dietary fat and risk of prostate cancer. *J. Natl. Cancer Inst.* 85:1571-1579 (1993).
12. Le Marchand, L., Kolonel, L.N., Wilkins, L.N., Myers, B.C., and Hirohata, T. Animal fat consumption and cancer of the prostate: a prospective study in Hawaii. *Epidemiology* 5:276-282 (1994).
13. Gann, P.H., Hennekens, C.H., Sacks, F.M., Grodstein, f., Giovannucci, E., and Stampfer, M.J. Prospective study of plasma fatty acids and risk of prostate cancer. *J. Natl. Cancer Inst.* 86:281-286 (1993).
14. Wang, Y., Corr, J.G., Thaler, H.T., Tao, Y., Fair, W.R., and Heston, W.D. Decreased growth of established human prostate LNCaP tumors in nude mice fed a low-fat diet. *J. Natl. Cancer Inst.* 87:1456-1462 (1995).
15. Chaudry, A.A., Wahle, K.W., McClinton, S. and Moffat, L.E. Arachidonic acid meabolism in benign and malignant prostatic tissue in vitro: effects of fatty acids and cyclooxygenase inhibitors. *Int. J. Cancer* 57:176-180 (1994).
16. Dahiya, R., Yoon. W.H., Boyle, B., Schoenberg, S., Yen, t.B. and Narayan, P. biochemical, cytogenetic and morphological characteristics of human primary and metastatic prostate cancer cell lines. *Biochem. Int.* 27:567-577 (1992).
17. Anderson, K.M., Wygodny, J.B., Ondrey, F., and Harris J. Human PC3 prostate cell line DNA synthesis is suppressed by eicosatetraynoic acid, an in vitro inhibitor of arachidonic acid metabolism. *Prostate* 12:3-12, 1988).
18. Ghosh, J. and Myers, C.E. Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem. biophys. Res. Commun.* 235:418-423 (1995).

19. Khan, W.A., Blobe, G.C., and Hannun, Y.A. Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. *Cell. Signaling* 7:171-184 (1995).
20. Schachter, J.B., Lester, D.S., and Alkon, D.L. Synergistic activation of protein kinase C by arachidonic acid and diacyglycerols in vitro: generation of a stable membrane-bound, cofactor-independent state of protein kinase c activity. *Biochim. Biophys. Acta.* 1291: 167-176 (1996).
21. Han, J., McCormick, F., and Macara, I.G. Regulation of rasGAP and the neurofibromatosis-1 gene product by eicosanoids. *Science* 252:576-579 (1991).
22. Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., and Lefkowith, J.B. Arachidonic acid metabolism. *Ann. Rev. Biochem.* 55:69-102 (1986).
23. Samuelsson, B., Dahlen, S.E., Lindgren, J.A., Rouzer, C.A., and Serhan, S.N. Leukotrienes and lipoxins: structures, biosynthesis and biological effects. *Science* 237:1171-1176 (1987).
24. Ara, G., and Teicher, B.A. Cyclooxygenase and lipoxygenase inhibitors in cancer therapy. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 54:3-16 (1996).
25. Young, M.R. Eicosanoids and he immunology of cancer. *Cancer Mets. Rev.* 13:337-348 (1994).
26. Nolan, R.D., Danilowicz, R.M., and Eling, T.E. Role of arachidonic acid metabolism in the mitogenic reponse of BALB/c 3T3 fibroblasts to epidermal growth factor. *Mol. Pharmacol.* 33:650-656 (1988).
27. Eling, T.E. and Glasgow, W.C. Cellular proliferation and lipid metabolism: importance of lipoxygenases in modulating epidermal growth factor-dependent mitogenesis. *Cancer Met. Rev.* 13:397-410 (1994).
28. Steller, H. Mechanisms and genes of cellular suicide. *Science* (Washington, DC) 267:1445-1449 (1995).
29. Lim, H., Paria, B.C., Das, S.K., Dinchuk, J.E., Langenbach, R., Trzaskos, J.M., and Dey, S.K. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* 91:197-208 (1997).
30. Goetzel, E.J., An, S., and Zheng, L. Specific suppression of prostaglandin E_2 of activation-induced apoptosis of human CD4+8+ T lymphoblasts. *J. Immunol.* 154:1041-1047 (1995).
31. Jaattela, M., Benedict, M., Teweri, M., Shayman, J.A., and Dixit, V.M. Bcl-x and Bcl-2 inhibit TNF and FAS-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene* 10:2297-2305 (1995).
32. O'Donnell, V.B., Spycher, S., and Azzi, A. Involvement of oxidents and oxident-generating enzyme(s) in tumor-necrosis-factor-alpha-mediated apoptosis: role for lipoxygenase pathway but not mitochondrial respiratory chain. *Biochem. J.* 310:133-141 (1995).

Massive, simultaneous translocation of Fas and FasL to the cell surface: a novel mechanism of cell death

Jagadananda Ghosh, Cheryl F. Murphy and Charles E. Myers, University of Virginia Cancer Center, Box 334, Charlottesville, Virginia 22908, USA.

Summary:

We have recently reported that human prostate cancer cells undergo massive apoptotic death when the metabolism of arachidonic acid through 5-lipoxygenase is interrupted (Ghosh and Myers (1998) *Proc. Natl. Acad. Sci. USA* **95:** 13482-13487). In our efforts to explore the molecular mechanisms underlying this massive, synchronous cell death we found that 5-lipoxygenase inhibition-induced death in prostate cancer cells involves Fas death receptor-mediated signaling and caspase activation. Untreated prostate cancer cells possess large intracellular pools of Fas and Fas ligand (FasL) with much smaller amounts on the surface. These cells, when treated with MK886, a specific inhibitor of arachidonate 5-lipoxygenase, showed a dramatic translocation of both Fas death receptor and FasL to the cell surface within 30 minutes of treatment, which could be prevented by exogenous addition of 5(S)-HETE, a product of 5-lipoxygenase. MK886 treatment increased the association of FADD (Fas-Associated Death Domain) and caspase 8 with the Fas receptor, and the enzymatic activity of caspase 8. Activation of caspase 8 was detected after 1-2 hours of MK886 treatment, whereas caspase 3-like activity was observed after 2-3 hours which peaked at 6-8 hours post treatment. Finally, blocking Fas death receptor-mediated signals with neutralizing anti-Fas or anti-FasL antibodies substantially reduced MK886-treated prostate cancer cell death. These findings suggest that there is an intact Fas death receptor-mediated signaling mechanism in human prostate cancer cells and that the metabolites of arachidonate 5-lipoxygenase play an unusual role regulating Fas/FasL-mediated signals to their programmed cell death.

Introduction:

Both epidemiologic studies and experiments with animal models have suggested that dietary fat might play a role as a risk factor for prostate cancer (1-7). Moreover, high fat diets have been found to be associated with advanced aggressive prostate cancers (8-10). Despite all of these studies, the molecular mechanisms by which dietary fat might influence the malignant behavior of prostate cells are incompletely understood. Previously, we reported that arachidonic acid, a common fatty acid in the Western style diet, can provide a proliferative stimulus to both hormone-responsive (LNCaP) and hormone-resistant (PC3) human prostate cancer cells *in vitro* through metabolic conversion to the 5-HETE series of eicosanoids via the action of 5-lipoxygenase (11).

We recently reported that human prostate cancer cells constitutively produce 5-HETE under serum-free conditions and that this increases dramatically in the presence of exogenous arachidonic acid. Inhibition of 5-lipoxygenase blocks 5-HETE production and induces massive apoptosis in both hormone-responsive and hormone-resistant human prostate cancer cells, suggesting a role of arachidonate 5-lipoxygenase in the survival of human prostate cancer cells (12). Moreover, inhibition of 5-lipoxygenase caused cell death despite the addition of androgen, epidermal growth factor or serum. Though 5-lipoxygenase plays a role in the survival and proliferation of human prostate cancer cells, a human breast cancer cell line, MDA-MB-231 was found to be completely refractory to the inhibition of this enzyme (13).

Apoptosis triggered by the inhibition of 5-lipoxygenase is unusual for cells of epithelial origin in several ways. Cell death following inhibition is quite rapid and occurs independent of the cell cycle. Within 45 minutes, mitochondrial permeability transition has occurred and at one-hour annexin-V binding to the cell surface is observed. Nucleosome formation appears at two hours and is complete by 6-8 hours. The pace of cell death is identical in the hormone-responsive LNCaP and in the hormone-resistant cell lines. These cell lines also differ in terms of the presence or absence of functional P53 or PTEN as well as baseline activation of NF-kappa -b, suggesting that the cell death pathway involved was independent of these factors.

Human prostate cancer cells have been reported to express Fas on their cells surface (14, 15) and when treated with drugs, were found to be sensitized to Fas-mediated death signals triggered when mFas L was presented by cytotoxic lymphocytes (16, 17). Moreover, a recent report showed that

prostate cells constitutively secrete active FasL molecules into the surrounding medium that can induce apoptosis in Fas-positive Ramos cells (18). Prostatic epithelial cells produce a bulk of the semen and they secrete considerable amounts of FasL into this fluid (19, 20). It has been speculated that the presence of FasL in semen help protect the sperm from an immune response on the part of the female (18), as production of FasL is a common property of immunoprivileged sites and some tumors (20, 21).

Since prostate cancer cells express both Fas and FasL in large amounts without undergoing spontaneous apoptosis, it is apparent that they must have some mechanism of preventing Fas activation or subsequent signaling events. However, nothing is known about the molecular regulation of Fas death receptor-mediated signaling in these cells in response to survival or proapoptotic agents. In the present report, we show that under control conditions, a vast majority of cellular Fas and FasL are sequestered intracellularly rather than being displayed on the cell surface. Following inhibition of 5-HETE production, there is rapid translocation of Fas and FasL to the cell surface followed by clustering of these molecules. This leads to the recruitment of FADD to Fas, followed by sequential activation of caspase 8 and caspase 3. Cell death is completely blocked by a dominant negative FADD construct as well as addition of an irreversible, cell permeant inhibitor of caspase 8. These findings indicate that 5-HETE specifically promotes survival of prostate cancer cells by blocking the translocation of Fas and FasL to the cell surface. Inhibition of 5-HETE synthesis triggers a form of apoptosis unique in cells of epithelial origin characterized by rapid, cell cycle-independent translocation of Fas and FasL to the cell surface. Moreover, this mechanism of cell death is independent of the presence or absence of functional P53 and active Akt/PKB. Various lipids have been shown to regulate cell growth and survival including LPA, ceramide, PPAR ligands, and various eicosanoids. It is apparent that the eicosanoid, 5-HETE, joins this list but functions via a mechanism distinct from another lipid regulator of cell survival.

Materials and Methods:

Cell culture and reagents: Androgen-responsive (LNCaP) and androgen-resistant (PC3) human prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). They were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) plus 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were fed every third day and split at a confluence of ~80%. MK886 was purchased from BIOMOL. Monoclonal anti-Fas and anti-FasL antibodies were obtained from Kamiya Biomedicals and Transduction

Laboratories respectively. Polyclonal antibodies to Fas and FasL were purchased from Santa Cruz Biotechnology. Caspase 8 monoclonal antibody (C15) was a kind gift from Dr. Carsten Scaffidi, Heidelburg, Germany. Monoclonal anti-FADD antibody was purchased from Transduction Laboratories.

Cell viability assay: Cell viability was determined using the MTS/PMS Cell Titer 96 AQ assay (Promega Corporation). Cells (3 x 10^3 per well) were plated in RPMI medium supplemented with 0.5% FBS in 96 well plates and incubated for 24 hours. Cells were pretreated either with anti-Fas (ZB4) or anti-FasL antibodies (0.5 μg/ml) for 24 hours and then treated with 4 μM MK886. Plates were incubated further for 48 hours after the addition of MK886. Control cells were treated with isotype matched nonspecific antibodies and the solvent vehicle. At the end of incubation period cell viability was measured by MTS/PMS cell titer assay (Promega Corporation) as previously described (11).

Flow cytometry: Cells (~3 x 10^5) were plated in RPMI medium 1640 supplemented with 10% FBS in 60 mm diameter dishes and allowed to grow for 48 hours. On the day of experiment the spent medium was replaced with 2 ml fresh serum-free RPMI medium 1640 and the cells were then treated with 10 μM MK886 for various periods of time (up to 4 hours). Control cells were treated with the madium containing solvent vehicle (0.02% DMSO). At the end of incubation periods, cells were harvested, washed and treated either with polyclonal anti-Fas or anti-FasL antibodies (1 μg/ml) for 60 minutes on ice. After brief washing cells were treated with 1 μg/ml fluorescent labeled secondary antibody (Goat anti-rabbit-FITC). Cells were then washed and analysed by a FACS Calibur flow cytometer (Beckton Dickinson).

Microscopy: Surface expression, and clustering of Fas death receptor and Fas ligand were studied in live prostate cancer cells by fluorescence microscopy using FITC-labeled monoclonal anti-Fas or anti-FasL antibodies with and without the inhibition of 5-lipoxygenase. PC3 human prostate cancer cells were seeded onto 25mm glass coverslips at a density of 500,000 per well in six well plates in RPMI medium supplemented with 10% FBS. The next day, the cells were treated with 75 μM MK886 for varying periods of time in culture medium at 37°C and simultaneously incubated with antibodies against Fas (Clone ANC95, non-death-inducing) or FasL (Clone H11) at a concentration of 5 μg/ml. Both the FITC-labeled antibodies were obtained from Alexis Biochemicals, San Diego, CA. Nuclei were stained with Hoechst 33342 (Molecular Probes,

Eugene, OR) at 10 μg/ml. At the end of the incubation periods, coverslips were rinsed in PBS briefly, and inverted onto a viewing chamber containing a small amount of culture medium without phenol red. Cells were visualized using a Zeiss Axioskop microscope equipped with appropriate fluorescence filter sets and a 63X Planapochromat oil-immersion objective. Grayscale images were collected with a SenSys CCD camera (Photometrics, Ltd., Tuscon, AZ.) using IPLab Spectrum software (Signal Analytics Corp., Vienna, VA.), and color coordinates merged. All image acquisition and manipulation were done using a Power Mac 9500.

Western blot: For Western blot, cells (~3 x 10^5) were plated in RPMI medium supplemented with 10% FBS in 60 mm diameter dishes and allowed to grow for 48 hours. The old medium was replaced with 2 ml fresh RPMI medium and the cells were treated with 10 μM MK886 for varying periods of time. After treatment, cells were harvested, washed and lysed by lysis buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk solution and then blotted with appropriate primary antibody followed by peroxidase-labeled secondary antibody. Bands were visualized by enhanced chemiluminescene (ECL) detection technique following manufacturer's instructions (Amersham Corporation).

Assay of caspases: Enzymatic activities of caspase 8 and caspase 3 were assayed using fluorescence-labeled specific tetrapeptide substrates in defined assay conditions (BIOMOL). Approximately 300,000 cells were plated in 60 mm diameter tissue culture plates in RPMI medium supplemented with 10% FBS and allowed to grow for 48 hours. On the day of experiments, the spent culture medium was replaced with 2 ml fresh serum-free RPMI medium 1640 and the cells were then treated with the 5-lipoxygenase inhibitor, MK886 (10 μM), for varying periods of time. At the end of incubation periods cells were lysed in caspase lysis buffer and aliquots of cell lysate (equivalent to 50 μg protein) were used for the assay of caspase activities.

DISC analysis: Formation of the death-inducing-signaling complexes were analysed by immunoprecipitation of Fas death receptor molecules followed by detection of FADD and caspase 8 by Western blot. Approximately 2 x 10^6 prostate cancer cells were plated in 100 mm diameter tissue culture plates (Falcon) in RPMI 1640 medium supplemented with 10% FBS. On the next day the old medium was replaced with 5 ml fresh serum-free RPMI medium and the cells were treated with 10 μM MK886 for 30-150 minutes at 37°C in the incubator. The cells were then harvested, washed and lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40 and a cocktail of protease inhibitors) for 45 minutes on ice. The lysates were

precleared with protein G-sepharose and the Fas death receptor complexes were immunoprecipitated using monoclonal anti-Fas antibody (IgG3) and protein G sepharose beads for 6 hours. Beads were then washed five times with the lysis buffer and then treated with sample buffer before separation by 15% SDS-PAGE. Association of FADD and caspase 8 with Fas death receptor was detected by Western blot using appropriate monoclonal antibodies.

Results:

Inhibition of 5-lipoxygenase triggers rapid translocation of Fas and FasL to the cell surface.

Under baseline conditions, prostate cancer cells express abundant Fas and FasL. A majority of both the molecules are sequestered intracellularly, with only a small proportion to be found on the cell surface. When prostate cancer cells were treated with MK886, a specific inhibitor of arachidonate 5-lipoxygenase, both Fas and FasL migrated to the plasma membrane in a time dependent manner and clustered. Within 15 minutes after inhibition of 5-lipoxygenase, an increase in cell surface FasL (mFasL) was apparent, while Fas translocation was apparent at 30 minutes post inhibition **(Figures 1a and 1b)**. By 30 minutes, clustering of both the molecules was apparent.

We analyzed the surface expression of Fas and FasL quantitatively. We observed that when prostate cancer cells, LNCaP (androgen-sensitive) and PC3 (androgen-resistant), were treated with MK886, there was a time dependent increase in the surface expression of Fas death receptor and Fas ligand **(Figures 1c, 1d)**, which were largely prevented by exogenous 5(S)-HETE, a product of 5-lipoxygenase.

To address the question of whether the increased surface expression of these molecules is associated with an increase in the amount of total proteins, we analysed Fas and FasL in whole cell lysates by Western blot, which showed that the amount of cellular FasL was found to be unchanged over a period of 4 hours. A minor increase in Fas was apparent at 3-4 hours post inhibition, but by then the cells were clearly apoptotic **(Figures 2a, 2b)**. Apoptosis was not inhibited by cycloheximide or actinomycin D, suggesting that this mechanism of cell death is largely independent of new protein synthesis (data not shown).

Inhibition of 5-lipoxygenase increases assembly of the elements of death-inducing

signaling complex (DISC).

When FasL binds with Fas death receptor a conformational change occurs in Fas molecule leading to trimerization of this receptor, which in turn leads to the recruitment of the adaptor protein FADD and procaspase 8. Prostate cancer cells showed a rapid increase in the association of FADD with Fas death receptor within 30 minutes of treatment with MK886 **(Fig 3a)**, coincident with the time when translocation of Fas to the cell surface and its clustering become apparent. Similar increase was also found in the association of procaspase 8 with Fas receptor complex **(Fig 3b)**. Interestingly, some background level of FADD and caspase 8 association with Fas molecule was also noticed in normal untreated cells.

MK886 treatment leads to an increase in the enzymatic activities of caspase 8 and caspase 3.

Caspase 8 is autoactivated when it associates with the Fas-FADD complex as procaspase 8. Caspase 8 is also called an initiator caspase which upon activation cleaves and activates other caspases, e.g., caspase 3, in the apoptotic cascade. When prostate cancer cells were treated with MK886, we observed a time dependent increase in the enzymatic activities of caspase 8 and caspase 3. Enhanced activity of caspase 8 was noticed within 1-2 hours **(Fig 4a)** while activation of caspase 3 was observed after 2-3 hours post treatment **(Fig 4b)**. This finding is consistent with other reports that activated caspase 8 (initiator caspase) cleaves and activates caspase 3 (the executioner caspase).

Pretreatment of the cells with a cell permeable irreversible inhibitor of caspase 8 dramatically inhibited MK886-induced apoptotic DNA degradation, documenting the critical role of this caspase in the cell death phenomenon **(Fig 4c)**. Similar inhibition of DNA degradation was also noticed when the cells were treated with brefeldin A, a well known blocker of protein translocation from endoplasmic reticulum (ER) to cell surface via Golgi apparatus (GA), suggesting an essential role of Golgi-mediated surface trafficking of Fas and FasL.

Blocking Fas/FasL-mediated signals lessed MK886-induced prostate cancer cell death.

To deliver a death signal through Fas receptor, FasL (soluble or membrane bound) needs to bind with Fas to make a complex. Any interruption of the binding of FasL to Fas death receptor might reduce the intensity or block Fas/FasL-mediated signal transduction. Fas itself, on the other hand, can undergo autoclustering and deliver apoptotic signals independent of the participation of its

ligand (22). To test the role of FasL, we pretreated the cells either with neutralizing anti-Fas (ZB4) or anti-FasL antibodies that hinder FasL binding with Fas before treating them with MK886. Our results showed that these blocking antibodies substantially reduced MK886-induced prostate cancer cell death, indicating that Fas/FasL-mediated signal transduction plays an active role in this type of programmed cell death **(Fig 5)**, though these antibodies failed to provide complete protection to the cells from MK886 treatment-induced death.

5-HETE regulates translocation of Fas and FasL from the Golgi.

MK886-induced prostate cancer cell death does not affect phosphorylation of Akt/PKB.

Phosphorylation and activation of Akt by phosphoinositide 3-kinase (PI3K) modulates a well known survival pathway. PI3-kinase, in turn, is dephosphorylated and inactivated by PTEN, (Phosphatase and Tensin homolog deleted on chromosome Ten), a lipid and protein phosphatase, that acts as a tumor suppressor. Mutation and inactivation of PTEN gene renders prostate cancer cells resistant to apoptosis by keeping PI3K constitutively activated that, in turn, phosphorylates and activates Akt. LNCaP cells lack functional PTEN resulting in constitutive activation of Akt. We observed that in LNCaP prostate cancer cells Akt phosphorylation is not affected during apoptosis triggered by inhibition of arachidonate 5-lipoxygenase **(Fig 6)**, indicating that this mechanism of cell death is independent of the activation state of Akt/PKB. Treatment of prostate cancer cells with wortmannin or LY294002 completely abolished phosphorylation of Akt in the same experimental conditions (J. Ghosh and C. E. Myers; unpublished observations).

Discussion:

Previously, we reported that human prostate cancer cells undergo massive apoptosis when they are deprived of 5-HETE, a metabolic product of arachidonate 5-lipoxygenase. A number of aspects of this apoptosis were unsual for cells of epithelial origin. Apoptosis was very rapid with nucleosome formation apparent as early as two hours after treatment with 5-lipoxygenase inhibitors. The onset of apoptosis was also independent of cell cycle progression. In seeking a molecular explanation underlying this rapid, synchronous mode of cell death, we chose to investigate the potential role of Fas death receptor because prostate cancer cells are known to constitutively express both Fas and FasL. Additionally, prostate cancer cells had been shown to release active, soluble FasL (sFasL) into the medium which could induce apoptosis in Fas-positive Ramos cells (18), a cell line known to be sensitive to sFasL-mediated signaling.

In this report, we document that under typical tissue culture conditions the human prostate cancer cell lines, PC3 and LNCaP, have large intracellular pools of Fas and FasL, but relatively little on the cell surface. This appears to account for the apparent resistance of these cells to endogenous Fas and FasL. After inhibition of arachidonate 5-lipoxygenase, FasL translocates to the cell surface within 15 minutes, followed by Fas at 30 minutes. These events were associated with recruitment of the adaptor protein, FADD, and procaspase 8 to Fas receptor, both apparent at 30 minutes. This is followed by activation of the caspase cascade and DNA degradation. Apoptosis was not inhibited by actinomycin D or cycloheximide and the rapid increase in the surface recruitment of Fas and FasL was not associated with a comparable increase in the total amount of these proteins, indicating that the triggering of prostate cancer cell death upon inhibition of arachidonate 5-lipoxygenase was largely due to surface translocation rather than *de novo* synthesis of these molecules. That cell death, in this case, is dependent on Fas activation is confirmed by the fact that anti-Fas and anti-FasL antibodies lessen cell death. This is further supported by the observation that inhibition of caspase 8 provides nearly complete protection to these cells from apoptosis.

These novel findings represent a unique example of simultaneous, rapid translocation of Fas and FasL from the cytosol to the membrane, triggering of which is regulated by the metabolites of arachidonate 5-lipoxygenase. Macrophages have been shown to possess large intracellular stores of FasL that is released following activation of these mononuclear cells. However, macrophages have not been described to have intracellular stores of Fas. Furthermore, in macrophages the FasL is released into the medium rather than arrayed on the cell surface as mFasL as it is in the prostate cancer cells. Finally, in macrophages this seems to be a mechanism facilitating attack on other cellular targets rather than an autocrine pathway for cell suicide. Though details of the molecular mechanisms underlying the triggering of Fas/FasL translocation to cell surface is not clear at this time, these findings document that in prostate cancer cells a functionally intact Fas/FasL-mediated death signaling mechanism is already in place and these cells are prone to autologous Fas/FasL-mediated suicide when forced with an appropriate trigger.

In the presence of DNA damaging cytotoxic drugs or other stimuli, wild-type p53 activates transcription of Fas and FasL. Recently, p53-mediated translocation of Fas from the cytosol to cell surface has been reported as a mechanism of rapid cell death (23), which apparently is independent of transcription. In prostate cancer, the absence or inactivation of p53 by mutation is associated with lower response rates to radiation or hormonal therapy, and is linked to a shortened patient

survival. The PC3 prostate cancer cell line is p53-negative and in LNCaP cells p53 is mutated, yet inhibition of 5-lipoxygenase still causes rapid Fas/FasL-mediated cell death, indicating that our findings represent a discrete example of p53-independent surface translocation of Fas and FasL leading to apoptotic cell death.

Phosphoinositide 3-kinase/Akt pathway plays an important role in prostate cancer biology by aiding resistance of this cancer to apoptosis. Apoptosis can be suppressed by activation of the serine/threonine kinase Akt/PKB by survival signals (24) such as insulin. Activation of Akt in turn phosphorylates and inactivates proapoptotic molecules like BAD, caspase 9 and FKHRL1, etc (25-28). Phosphorylated Akt regulates FasL expression through FKHRL and Fas receptor signaling, and thus mediates cell survival. In LNCaP prostate cancer cells Akt is known to be constitutively phosphorylated, consistent with the previous reports of mutational inactivation of PTEN, a lipid and protein phosphatase (29-31). PC3, on the other hand, possessed wild type PTEN and Akt exhibits a low level of phosphorylation under standard tissue culture conditions. Yet both the cell lines showed rapid Fas and FasL translocation to the cell surface followed by cell death. Thus, inhibition of arachidonate 5-lipoxygenase triggers prostate cancer cell death without regard to the phosphorylation state of Akt, indicating that this kind of apoptosis is able to overcome the negative regulation of the apoptosis machinery by PKB/Akt-mediated survival signaling.

Both Fas and FasL are expressed in normal prostate tissue and appear to play a role in prostate gland biology. FasL is secreted into the semen which may play an important role in protecting the sperm cells from immune cell attack during their sojourn in the female reproductive organs. Coexpression of Fas and FasL are characteristic of epithelial tissues with high turnover, including prostate tissue. Normal prostate tissue undergoes apoptosis when androgens are withdrawn and this process is associated with increased expression of Fas and FasL, although the importance of these events remained controversial. However, despite a growing literature on Fas/FasL in the biology of normal and cancerous prostate tissue, the existing literature yields no evidence for the unusual function of Fas and FasL seen in the current study. These facts suggest that studies of the subcellular distribution of these two molecules in prostate cells in the presence and absence of androgen may be warranted.

The role of dietary fat in the development and progression of prostate cancer is still a controversial issue. Epidemiologic studies yield a conflicting picture of the role of dietary fat in prostate cancer. In contrast, in tissue culture conditions and animal models, arachidonic acid has been shown to

have a profound impact on prostate cancer biology. The expression of arachidonate 12-lipoxygenase characterizes advanced disease with a poor prognosis. Tumor cell invasion and angiogenesis are stimulated by the arachidonate 12-lipoxygenase product, 12-HETE (32). Conversion of radiolabeled arachidonic acid to prostataglandin E2 is ten times higher in prostate cancer than in the surrounding normal tissues (33). These observations set the context of our findings that another arachidonic acid product, 5-HETE, plays a central role in regulating subcellular distribution and function of the Fas death receptor and its ligand. Taken together, these findings provide a powerful biochemical rationale for why a diet rich in omega-6 fatty acids might favor the progression of prostate cancer.

Mice in whom the arachidonate 5-lipoxygenase gene is knocked out are fertile and have a normal life span (34, 35). In prostate cancer cells, inhibition of this enzyme triggers cell death by an unusual mechanism involving rapid translocation of Fas and FasL from intracellular stores to the cell surface followed by activation of caspase 8. Additionally, this form of apoptosis appears to take place despite the inactivation of p53 and constitutive activation of cAkt, as well as the presence of androgen and serum growth factors in the medium. These facts suggest that the pathway linking 5-HETE to subcellular localization of Fas and FasL may represent a useful target for prostate cancer drug development.

The cause of cancer-related tumor burden is excessive cell proliferation and decreased cell death, which results in a state of defunct tissue homeostasis. Chemotherapeutic drugs are designed to reverse the order of cancer cell behavior blocking cell division and increasing cell death, resulting in net loss of cell number. Drugs currently in use for cancer chemotherapy, in most cases, work by induction of apoptosis, either by the way of activation of CD95 (Fas)-mediated signals or by the involvement of mitochondria. Tumor cells, on the other hand, are posing a state of drug resistance by deregulating induction of the death program ("drug neo-resistance") or immunoresistanece by expressing FasL on their surface to counterattack the anti-tumor lymphocytes. Prostate cancer cells are equipped with both these arsenals and are very difficult to be managed by chemotherapeutic agents, at reasonably low concentrations to be used clinically. Loss of sensitivity to death signals may play a role in the progression of human prostate cancer, and our recent finding that inhibition of arachidonate 5-lipoxygenase triggers autocrine Fas/FasL-mediated prostate cancer cell death, opens up a new direction of medical research involving regulation of Fas death receptor-mediated signals in the chemotherapy of human malignancies.

References:

1. Kondo, Y., Homma, Y., Aso, Y., and Kakizoe, T. (1994) Promotional effect of two-generation exposure to a high-fat diet on prostate carcinogenesis in ACI/Seg rats. Cancer Res. 54, 6129.
2. Meikle, A. W., and Smith, J. A., Epidemiology of prostate cancer. Urol. Clin. North America 17: 709-718, 1990.
3. Wang, Y., Corr, J. G., Thaler, H. T., Tao, Y., Fair, W. R., and Heston, W. D., Decreased growth of established prostate LNCaP tumors in nude mice fed a low-fat diet. Journal of National Cancer Institute 87: 1456-1462, 1995.
4. Ross, R. K., and Henderson, B. E., Do diet and androgens alter prostate cancer risk via a common etiologic pathway. Journal of National Cancer Institute. 86: 252-254, 1994.
5. Le, M. L., Kolonel, L. N., Wilkens, L. R., Myers, B. C., and Hirohata, T. (1994) Animal fat consumption and prostate cancer: a prospective study if Hawaii [see comments]. Epidemiology 5: 276-282.
6. Franceschi, S., Fat and prostate cancer. Epidemiology [Editorial; comment] 5: 271-273, 1994.
7. Brawley, O. W., and Thompson, I. M., Chemoprevention of prostate cancer. Urology 43: 594-599, 1994.
8. West, D. W., Slattery, M. L., Robison, L. M., French, T. K., and Mahoney, A. W., Adult dietary intake and prostate cancer risk in Utah: a case control study with special emphasis on aggressive tumors. Cancer Causes Control 2: 85-94, 1991.
9. Giovannucci, E., Rimm, E. B., Colditz, G. A., Stampfer, M. J., Ascherio, A., Chute, C. C., and Willett, W. C., A prospective study of dietary fat and risk of prostate cancer. Journal of National Cancer Institute 85: 1571-1579, 1993.
10. Gann, P. H., Hennekens, C. H., Sacks, F. M., Grodstein, F., Giovannucci, E. L., and Stampfer, M. J., Prospective study of plasma fatty acids and risk of prostate cancer. Journal of National Cancer Institute 86: 281-286, 1994.
11. Ghosh, J., and Myers, C. E. (1997) Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. Biochem Biophys Res. Commun. 235: 418-423.
12. Ghosh, J., and Myers, C. E. (1998) Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. Proc. Natl. Acad. Sci. USA 95, 13182-13187.
13. Ghosh, J., and Myers, C. E. (1999) Central role of arachidonate 5-lipoxygenase in the

regulation of cell growth and apoptosis in human prostate cancer cells. Adv. Exp. Med. Biol. 469, 577-582.

14. Rokhlin, O. W., Bishop, G. A., Hostager, B. S., Waldschmidt, T. J., Sidorenko, S. P., Pavloff, N., Kiefer, M. C., Umansky, S. R., Glover, R. A., and Cohen, M. B. (1997) Fas-mediated apoptosis in human prostatic carcinoma cell lines. Cancer Res. 57, 1758-1768.
15. Hedlund, T. E., Duke, R. C., Schleicher, M. S., and Miller, G. J. (1998) Fas-mediated apoptosis in seven human prostate cancer cell lines: correlation with tumor stage. Prostate 36, 92-101.
16. Frost, P. J., Ng, C. P., Belldegrun, A., and Bonavida, B. (1997) Immunosensitization of prostate carcinoma cell lines for lymphocytes (CTL, TIL, LAK)-mediated apoptosis via the Fas-FasL pathway of cytotoxicity. Cellular Immunol. 180, 70-83.
17. Uslu, R., Borsellino, N., Frost, P. J., Garban, H., Ng, C. P., Mizutani, Y., Belldegrun, A., and Bonavida, B. (1997) Chemosensitization of human prostate carcinoma cell lines to anti-Fas-mediated cytotoxicity and apoptosis. Clin. Cancer Res. 3, 963-972.
18. Liu, Q. Y., Rubin, M. A., Omene, C., Lederman, S., and Stein, C. A. (1998) Fas ligand is constitutively secreted by prostate cancer cells in vitro. Clin. Cancer Res. 4, 1803-1811.
19. Xerri, L., Devilard, E., Hassoun, J., Mawas, C., and Birg, F. (1997) Fas ligand is not only expressed in immune privileged human organs but is also coexpressed with Fas in various epithelial tissues. Mol. Pathol. 50, 87-91.
20. Hahne, M., Rimoldi, D., Schroter, M., Romero, P., Schreier, M., French, L., Schneider, P., Bornand, T., Fontana, A., Leinard, D., Cerottini, J-C., and Tschopp, J. (1996) Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumor immune escape. Science (Washington DC) 274, 1363-1366.
21. Griffith, T., Brunner, T., Fletcher, S., Green, D., and Ferguson, T. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. Science (Washington DC) 270, 1189-1192
22. Beltinger, C., Fulda, S., Kammertoens, T., Meyer, E., Uckert, W., and Debatin, K-M. (1999) Herpes simplex virus thymidine kinase/gancyclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. Proc. Natl. Acad. Sci. USA. 96, 8699-8704.
23. Bennett, M., Macdonald, K., Chan, S-W., Luzio, J. P., Simari, R., and Weissberg, P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-induced apoptosis. Science 282, 290-293.

24. Dudek, H., Dutta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Regulation of neuronal survival by the serine-threonine kinase Akt. Science 275, 661-665.

25. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science 282, 1318-1321.

26. Hausler, P., Papoff, G., Eramo, A., Reif, K., Cantrell, D. A., and Ruberti, G. (1998) Protection of CD95-mediated apoptosis by activation of phosphatidylinositide 3-kinase and protein kinase B. Eur. J. Immunol. 28, 57-69.

27. Dutta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231-241.

28. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. J., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., And Greenberg, M. E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription. Cell 96, 857-868.

29. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. (1998) The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA 95, 15587-15591.

30. Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J., and Roth, R. A. (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J. Biol. Chem. 274, 21528-21532.

31. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., et al. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275, 1943-1947.

32. Nie, D., Hillman, G. G., Geddes, T., Tang, K., Pierson, C., Grignon, D. J., and Honn, K. V. (1998) Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. Cancer Research 58, 4047-4051.

33. Dahiya, R., Yoon, W. H., Boyle, B., Schoenberg, S., and Narayan, P. (1992) Biochem. Int. 27, 567-577.

34. Chen, X., Sheller, J., Johnson, E., and Funk, C. D. (1994) Nature (London) 372, 179-182.

35. Funk, C. D., Kurre, U., and Griffis, G. (1994) Ann. N. Y. Acad. Sci. 714, 252-258.

Legends

Figure 1. Surface Translocation and Clustering of Fas and FasL in Prostate Cancer Cells Upon Treatment With MK886.

For microscopy PC3 prostate cancer cells were plated in RPMI medium 1640 supplemented with 10% FBS for 24 hours on cover glasses and treated with MK886 for various periods of time. Figures 1 a (i) and 1 b (i) show fixed control PC3 cells permeabilized and stained for Fas and Fas ligand, respectively, to reveal the total amount of each protein present in the cells. Live intact cells were incubated either with FITC labeled monoclonal anti-Fas, (a), or anti-FasL, (b), antibodies (Alexis), mounted on glass slides and observed under fluorescent microscope as described in the methods section. a, b (ii) Control cells; a (iii) cells treated with MK886 for 30 minutes; a (iv) cells treated with MK886 for 75 minutes; b (iii) cells treated with MK886 for 15 minutes; b (iv) cells treated with MK886 for 30 minutes.

PC3 and LNCaP prostate cancer cells were grown for 48 hours in RPMI medium 1640 in 60 mm diameter tissue culture dishes and treated with 10 μM MK886 for various periods of time with and without the addition of 5(S)-HETE (500 nM). Cells were then harvested and treated either with polyclonal anti-Fas (c) or anti-FasL (d) antibodies for 1 hour on ice. Cells were pelleted down, resuspended and treated with secondary goat anti-rabbit antibody labeled with FITC for 45 minutes on ice. Cells were analysed by FACS Calibur flow cytometer (Beckton-Dickinson).

Figure 2. Protein Levels of Fas and FasL in Prostate Cancer Cells After Treatment With MK886. LNCaP prostate cancer cells were grown for 48 hours in 60 mm diameter culture dishes in RPMI medium 1640. The spent medium was replaced with fresh 2 ml serum-free RPMI medium 1640 and the cells were treated with 10 μM MK886 for varying periods of time [hours]. At the end of incubation periods cells were harvested, washed and lysed in lysis buffer. Proteins (50 μg per lane) were separated by 15% SDS-PAGE and transferred to nitrocellulose paper. Expression levels of Fas (a) and FasL (b) were determined by Western blot using rabbit polyclonal anti-Fas (Santa Cruz) or monoclonal anti-FasL (Transduction Laboratories) antibodies respectively.

Figure 3. Assembly of Death-inducing Signaling Complex (DISC).
PC3 prostate cancer cells were plated in 100 mm diameter culture dishes and allowed to grow for

48 hours. On the day of experiments the spent medium was replaced with 5 ml fresh serum-free RPMI medium 1640 and the cells were treated with 10 μM MK886 for various periods of time [minutes]. At the end of incubation periods cells were harvested, washed and lysed in lysis buffer. Cell lysates were precleared with protein G-sepharose and the Fas receptor complexes were immunoprecipitatead using monoclonal anti-Fas antibody (APO-1-3, Kamiya) and protein G-sepharose for 6 hours. The beads were washed five times in lysis buffer and treated with SDS sample buffer. Immunecomplexes were resolved by 15% SDS-PAGE, transferred to nitrocellulose paper and blotted with monoclonal anti-FADD (a) or monoclonal anti-caspase 8 (b) antibodies. Bands were visualized by ECL detection technique.

Figure 4. Activation of Caspase 8 and Caspase 3.
LNCaP prostate cancer cells were plated in 60 mm diameter plates as described in figure 2 above and treated with MK886 for times as indicated. Cells were then harvested and lysed in caspase lysis buffer. Aliquots of cell lysates equivalent to 50 μg proteins were taken and caspase enzymatic activities determined with an assay kit following manufacturers instructions (BIOMOL), (A) activation of caspase 8 and (B) activation of caspase 3.

Figure 5. Prevention of Cell Death by Neutralizing anti-Fas-mediated Signals.
LNCaP prostate cancer cells (3×10^3 per well) were plated for 24 hours in 96 well tissue culture plates in RPMI medium supplemented with 0.5% FBS. Cells were then pretreated with anti-Fas (ZB4) or anti-FasL antibodies (0.5 μg/ml) for 24 hours before the addition of MK886 (4 μM). Control cells were treated with isotype matched control antibodies and the solvent vehicle. Plates were further incubated at 37°C for 48 hours in the incubator after the addition of MK886. Cell viability was determined by MTS/PMS cell titer assay (Promega Corporation).

Figure 6. Phosphorylation State of Akt/PKB
LNCaP prostate cancer cells were plated as in figure 2 and treated with MK886 for different time periods. Cells were then harvested, lysed and proteins (50 μg per lane) were separated by 15% SDS-PAGE. Proteins were transferred to nitrocellulose paper and then blotted with polyclonal anti-phospho-Akt (specific for Ser-473) antibody obtained from New England Biolabs. Bands were visualized by ECL detection technique. Amounts of Akt proteins in each lane was determined using monoclonal anti-Akt antibody from the same supplier.

Appendix 4

RESEARCH PROJECT 7

"Survival Signaling and Prostate Cancer"

Charles E. Myers, Michael Weber And George Kulik

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

In metastatic prostate cancer, androgen withdrawal results in a complete response in less than 10% of men. Pathologic examination of tumor samples shows that only a minority of the cancer cells undergo apoptosis, while the majority simply experience growth arrest. Resistance to therapy-induced apoptosis is likely also to be a major impediment to successful treatment with chemotherapy or radiation.

We and others have shown that prostate cancer cells express several redundant "survival signaling" pathways that render them resistant to androgen withdrawal and pro-apoptotic therapies. These include (*1*) overexpression of bcl family members; (*2*) absence of PTEN with constitutive activation of Akt; (*3*) autocrine stimulation of EGF receptor and increased expression of HER2; and (*4*) increased production of neuropeptides that activate PKA, which in prostate cancer cells stimulates survival. The hormone-responsive cell line, LNCaP, exhibits all of these alterations save for increased bcl-2 expression.

In **Specific AIM I**, we will test the ability of inhibitors of each of these survival pathways singly and in combination for their ability to sensitize cells to androgen withdrawal and pro-apoptotic therapies. In **Specific Aim II** the combination of therapies that best induces apoptosis *in vitro* will be tested in murine xenograft models. *In vivo* the endpoints will be frequency of complete remission, normalization of PSA, and frequency of apoptosis in tumor samples. During these experiments, we will also adapt the newly developed ^{99}Tc-annexin V technique to image apoptosis non-invasively *in vivo*.

The core of this proposal is the concept that increased apoptosis in prostate cells can be achieved by a combination of anti-survival and pro-apoptotic drugs. Because they target different mechanisms we expect that such a combination will have strong synergistic effects and increase apoptosis without increasing toxicity. We expect these findings to guide the design of a clinical trial.

PERFORMANCE SITE(S) (*organization, city, state*)

University of Virginia
Health System
Charlottesville, VA 22908

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Charles E. Myers, M.D.	University of Virginia	Co-Investigator
Michael J. Weber, Ph.D.	University of Virginia	Co-Investigator
George Kulik, Ph.D.	University of Virginia	Co-Investigator

DD Project 7 Myers et al — Principal Investigator/Program Director *(Last, first, middle):* Myers, Charles E.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/00 — THROUGH 06/30/01

PERSONNEL *(Applicant organization only)* NAME	ROLE ON PROJECT	TYPE APPT. *(months)*	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED *(omit cents)* SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Myers, Charles E., M.D.	co-investigator	12	10	141,300	14,130.	3,321.	17,451.
Weber, Michael J. Ph.D.	co-investigator	12	5	130,100	6,505.	1,529.	8,034.
Kulik, George Ph.D.	co-investigator	12	30	38,900	11,670.	2,742.	14,412.
Murphy, Cheryl	Senior Lab Technician	12	50	48,282	24,141.	8,087.	32,228.
Hensley, Renea	Laboratory technician	12	50	25,000	12,500.	4,188.	16,688.
SUBTOTALS →					68,946.	19,866.	88,813.

CONSULTANT COSTS				0.
EQUIPMENT *(Itemize)*				0.
SUPPLIES *(Itemize by category)*				
Purchase of mice (parents)			4,460.	
Tissue culture supplies and drugs			10,000.	
				14,460.
TRAVEL				0.
PATIENT CARE COSTS	INPATIENT	None	0.	0.
	OUTPATIENT	None	0.	0.
ALTERATIONS AND RENOVATIONS *(Itemize by category)*			0.	
			0.	0.
OTHER EXPENSES *(Itemize by category)*				
Per diem expenses to raise mice			22,140.	
				22,140.
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD				**$ 125,413.**
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS			0.
	FACILITIES AND ADMINISTRATION COSTS			0.
TOTAL DIRECT CO			*ice Page)* →	**$ 125,413.**

PHS 398 (Rev. 4/98) — DD
Number pages consecutivel — such as

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD *(from Form page 4)*	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits* *Applicant organization only*		88,813.	90,954.	93,159.	95,375.	97,452.
CONSULTANT COSTS		0.	0.	0.	0.	0.
EQUIPMENT		0.	0.	0.	0.	0.
SUPPLIES		14,460.	14,894.	15,341.	15,801.	16,275.
TRAVEL		0.	0.	0.	0.	0.
PATIENT CARE COSTS	INPATIENT	0.	0.	0.	0.	0.
	OUTPATIENT	0.	0.	0.	0.	0.
ALTERATIONS AND RENOVATIONS		0.	0.	0.	0.	0.
OTHER EXPENSES		22,140.	22,804.	23,488.	24,193.	24,919.
SUBTOTAL DIRECT COSTS		125,413.	128,652.	131,988.	135,369.	138,646.
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	0.	0.	0.	0.	0.
	F & A	0.	0.	0.	0.	0.
TOTAL DIRECT COSTS		125,413.	128,652.	131,988.	135,369.	138,646.

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face* → **$ 660,068.**

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Please see following page for budget justification.

Budget Justification

Personnel:

Charles E. Myers, M.D. (10% effort) Dr. Myers will supervise the tissue culture and animal model experiments. Dr Myers has extensive experience in the area of preclinical drug development including synergy and anti-agonism experiments and in vivo testing of cancer drug efficacy. His laboratory discovered the pro-apoptotic effects of inhibiting lipoxygenase synthesis and the rol of FAS in that process.

Michael J. Weber, Ph.D. (5% effort) Dr. Weber has recently published extensively on the survival pathways activated in human prostate cancer with a special emphasis on the LNCaP cell line. He will provide expert advice to Drs. Myers and Kulik in the conduct of the experiments outlined in this grant application.

George Kulik, Ph.D. (30% effort) Dr. Kulik has worked in Dr. Weber's laboratory on the characterization of survival pathways active in human prostate cancer cell lines. He will determine the impact of the various drugs to be tested on the activity of the key survival pathways that play a role in prostate cancer survival. Should blockade of all known survival pathways fail to induce complete remissions in LNCaP in vivo, Dr. Kulik and Dr. Weber will investigate the presence of novel survival pathways htat might account for these results.

Cheryl Murphy (50% effort): Ms. Murphy is the senior technician on these experiments and will be responsible for supervising the execution of the tissue culture and animal experiments.

Renea Hensley (50% effort): Ms. Hensley will work under the supervision of Ms. Murphy and will aid in the conduct of the tissue culture and animal experiments.

Equipment:

None requested

Supplies and Other Expenses:

The major non-personnel expense is for the animal experiments. We anticipate the use of 600 mice per year, kept for 60 days. This will permit two full sets of combination dose response curves to be performed per year, with 10 mice per group, assuming that sample mice will have to be sacrified periodically to examine apoptosis induction. We request $4,460 for the purchase of parent mice, and estimate that the per diem expense of raising mice will come to $22,140 in year one. We also request $10,000 for the purchase of tissue culture supplies and drugs.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Charles E. Myers, Jr., M.D.	Director, Cancer Center, University of Virginia

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Wesleyan University, Middletown Connecticut	B.A.	1965	Medicine
University of Pennsylvania, Philadelphia, PA	M.D.	1969	

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

PROFESSIONAL EXPERIENCE:

Internship and Residency, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania	1967-1970
Clinical Associate, Medicine Branch, National Cancer Institute, Bethesda, Maryland	1971-1972
Clinical Assoc., Laboratory of Chemical Pharmacology, National Cancer Institute, Bethesda, MD	1972-1974
Senior Investigator, Medicine Branch, National Cancer Institute, Bethesda, Maryland	1974-1975
Senior Investigator (joint appointment), Medicine Branch, National Cancer Institute, Bethesda, MD	1976-1977
Head, Biochemical Pharmacology, Clinical Pharmacology Branch, and Attending Physician, Medicine Branch, National Cancer Institute, Bethesda, Maryland	1978-1994
Chief, Clinical Pharmacology Branch, National Cancer Institute, Bethesda, Maryland	1981-1982
Consulting Attending, NCI-Navy Oncology	1982-1983
Chief, Medicine Branch, National Cancer Institute, Bethesda, Maryland	1988-1990
Chief, Clinical Pharmacology Branch, National Cancer Institute, Bethesda, Maryland	1990-1994
Director, Cancer Center, University of Virginia, Charlottesville, VA	1994-present

HONORS AND AWARDS:

Alpha Omega Alpha (1967), Junior Year
Rittenhouse Book Store Award for excellence in Medicine (1969)
Elected to American Society for Clinical Investigation (1981)
Pfizer Award Lecture (1981)
Assistant Program Chairman, Gordon Conference on Chemotherapy (1984)
Program Chairman, Gordon Conference on Chemotherapy (1985)
Milken Foundation Award (1988)

PUBLIC HEALTH SERVICE PROMOTIONS, CITATIONS, AWARDS:

Promotion to Senior Surgeon 05 (1975)
PHS Commendation Medal (1979)
Promotion to 06 (1981)
PHS Unit Commendation (1988)

MEDICAL LICENSE:

State of Pennsylvania – 1970
Commonwealth of Virginia – 5/94

BOARD CERTIFICATION:

Diplomate, American Board of Internal Medicine – 1973
Diplomate, American Board of Oncology – 1975

SELECTED PUBLICATIONS from a total of 248

Turk, T.M.T., Rees, M.A., Pietrow, P., **Myers, C.E.**, Mills S.E., Gillenwater, J.Y. Determination of optimal freezing parameters of human prostate cancer in a nude mouse model. *Prostate.* 38(2):137-143, 1999 Feb.

Myers, C.E., Ghosh, J. Lipoxygenase inhibition in prostate cancer. *European Urology.* 35(5-6):395-398, 1999 May-Jun.

Myers, C.E., Differentiating agents and nontoxic therapies. *Urologic Clinics of North America.* 26(2):341-, 1999 May.

Bergan, R.C., Reed, E., **Myers, C.E.**, Headlee, D., Brawley, O., Cho, H.K., Tompkins, A., Lineham, W.M., Kohler, D., Steinberg, S.M., Blagosklonny, M.V. A phase II study of high-dose Tamoxifen in patients with hormone-refractory prostate cancer. *Clinical Cancer Research.* 5(9):2366-2373, 1999 Sept.

Bubley, G.J., Carducci, M., Dahut, W., Dawson, N., Daliani, D., Eisenberger, M., Figg, W.D., Freidlin, B., Halabi, S., Hudes, G., Hussain, M., Kaplan, R., **Myers, C.E.**, Oh, W., Petrylak, D.P., Reed, E., Roth, B., Sartor, O., Scherr, H., Simons, J., Sinabaldi, V., Small, E.J., Smith, M.R., Trump, D.L., Vollmer, et al. The eligibility and response guidelines for phase II clinical trials in androgen-independent prostate cancer. Recommendations from the prostate-specific antigen working Group. *Journal of Clinical Oncology.* 17(11):3461-3467, 1999 Nov.

Larner, J., Jane, J., Laws, E., Packer, R., **Myers, C.E.**, Shaffrey, M. A phase I-II trial of lovastatin for anaplastic astrocytoma and glioblastoma. *American Journal of Clinical Oncology-Cancer Clinical Trials.* 21(6):579-583.

Thibault, A., Figg, W.D., Bergan, R.C., Lush, R.M., **Myers, C.E.**, Tompkins, A., Samid, D. A phase II study of 5-AZA-2'Deoxycytidine (Decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori.* 84(1):87-89, 1998.

J. Ghosh and **C. E. Myers**. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl. Acad. Sci. USA.* Vol 95, pp. 13182-13187, 1998.

N.A. Dawson, W.D. Figg, M.R. Cooper, O. Sartor, R.C. Bergan, A.M. Senderowicz, S.M. Steinberg, A. Tompkins, B. Weinberger, E.A. Sausville, E. Reed and **C.E. Myers**. Phase II trial of suramin, leuprolide, and flutamide in previously untreated metastatic prostate cancer. *J Clin Oncol.* 15: p. 1470-7, 1997.

J. Ghosh and **C.E. Myers**. Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem Biophys Commun.* 235: p. 418-23, 1997.

P.L. de Souza, M. Castillo and **C.E. Myers**. Enhancement of paclitaxel activity against hormone-refractory prostate cancer cells in vitro and in vivo by quinacrine. *Br J Cancer.* 75: p. 1593-600, 1997.

C.J. Bowden, W.D. Figg, N.A. Dawson, O. Sartor, R.J. Bitton, M.S. Weinberger, D. Headlee, E. Reed, **C.E. Myers** and M.R. Cooper. A phase I/II study of continuous infusion suramin in patients with hormone-refractory prostate cancer: toxicity and response. *Cancer Chemother Pharmacol.* 39: p. 1-8, 1996.

A.C. Dalkin, J.T. Gilrain, D. Bradshaw **and C.E. Myers**. Activin inhibition of prostate cancer cell growth: selective actions on androgen-responsive LNCaP cells. *Endocrinology.* 137: p. 5230-5, 1996.

R. Danesi, D. Nardini, F. Basolo, T.M. Del, D. Samid, **C.E. Myers**. Phenylacetate inhibits protein isoprenylation and growth of the androgen-independent LNCaP prostate cancer cells transfected with the T24 Ha-ras oncogene.*Mol Pharm.* 49(6): p. 972-9, 1996.

R. Danesi, C.A. McLellan and **C.E. Myers**. Specific labeling of isoprenylated proteins: application to study inhibitors of the post-translational farnesylation and geranylgeranylation. *Biochem biophys Res Commun.* 206(2): p. 637-43, 1995.

R. Danesi, W.D. Figg, E. Reed and **C.E. Myers**. Paclitaxel (taxol) inhibits protein isoprenylation and induces apoptosis in PC-3 human prostate cancer cells. *Mol Pharmacol.* 47(6): p. 1106-11, 1995.

W.D. Figg, O. Sartor, M.R. Cooper, A. Thibault, R.C. Bergan, N. Dawson, E. Reed and **C.E. Myers**. Prostate specific antigen decline following the discontinuation of flutamide in patients with stage D2 prostate cancer. *Am J Med.* 98(4): p. 412-4, 1995.

M.M. Borner, **C.E. Myers**, O. Sartor, Y. Sei, T. Toko, J.B. Trepel and E. Schneider. Drug-induced apoptosis is not necessarily dependant on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3. *Cancer Res.* 55(10): p. 2122-8, 1995.

S.C. Piscitelli, A. Thibault, W. D. Figg, A. Tompkins, D. Headlee, R. Lieberman, D. Samid, and **C.E. Myers**. Disposition of phenyl- butyrate and its metabolites, phenylacetate and phenylacetyglutamine. *J Clin Pharamacol.* 35(4): p. 368-73, 1995.

R.J. Bitton, W.D. Figg, D.J. Venzon, M.C. Dalakas, C. Bowden, D. Headlee, E. Reed, **C.E. Myers** and M.R. Cooper. Pharmacologic variables associated with the development of neurologic toxicity in patients treated with suramin. *J Clin Oncol.* 13(9): p. 2223-9, 1995.

W. R. Hudgins, S. Shack, **C.E. Myers** and D. Samid. Cytostatic activity of phenylacetate and derivatives against tumor cells. Correlation with lipophilicity and inhibition of protein prenylation. *Biochem Pharmacol.* 50(8): p. 1273-9, 1995.

Thibault, D. Samid, M.R. Cooper, W.D. Figg, A.C. Tompkins, N. Patronas, D.J. Headlee, D.R. Kohler, D.J. Venzon **and C.E. Myers**. Phase I study of phenylacetate administered twice daily to patients with cancer. *Cancer.* 75(12): p. 2932-8, 1995.

L. Whitesell, E.G. Mimnauger, C.B. De, **C.E. Myers** and L.M. Neckers. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: Essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci USA.* 91(18): p. 8324-8, 1994.

D. Samid, Z. Ram, W.R. Hudgins, S. Shack, L. Liu, S. Walbridge, E.H. Oldfield and **C.E. Myers**. Selective activity of phenylacetate against malignant gliomas: resemblance to fetal brain damage in phenylketonuria. *Cancer Res.* 54(4): p. 891-5, 1994.

D. Samid, S. Shack and **C.E. Myers**. Selective growth arrest and phenotypic reversion of prostate cancer cells in vitro by nontoxic pharmacological concentrations of phen

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Michael J. Weber	Professor of Microbiology

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Haverford College, Haverford PA	B.Sc.	1963	Biology
Univ. California, San Diego CA	Ph.D.	1968	Cell Biology
Univ. California, Berkeley CA	Postdoc.		Virology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional and Research Experience:

Postdoctoral with Harry Rubin, Virus Laboratory, Univ. of California, Berkeley, 1968-1970.
Assistant Professor of Microbiology, University of Illinois, 1970-1975.
Associate Professor of Microbiology, University of Illinois, 1975-1982.
On leave at the Imperial Cancer Research Fund, London, England in the laboratory of Dr. John Wyke, 1979.
Professor of Microbiology, University of Illinois, 1982-1984.
Frank Talbot Visiting Professor at the Univ. of Virginia School of Medicine, 1983-1984.
Professor of Microbiology, Univ. of Virginia School of Medicine, 1984-present.
Director, Molecular Medicine Graduate Program, 1993-present
Associate Director for Laboratory Research, University of Virginia Cancer Center, 1994-present.

Academic and Professional Honors:

Dernham Post-Doctoral Fellow of the American Cancer Society.
Research Career Development Award, 1976-1980.
List of Teachers Ranked as Excellent, University of Illinois, Fall 1980.
Member, Molecular Biology Study Section, NIH, 1977-1981.
Frank Talbot Visiting Professor at the University of Virginia, 1983-1984.
Member, American Cancer Society Study Section, Pers. B. 1986-1989.
Merit Award for NCI R01-CA39076, "Signal transmission by the *src* oncogene."
Editor, *Molecular and Cellular Biology*, 1992-1997.
Weaver Professor of Oncology, 1997-present

Representative Recent Publications:

Samuels, M.L., Weber, M.J., Bishop, J.M., and McMahon, M. 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradio-dependent human Raf-1 protein kinase. *Mol. Cell. Biol.* **13:**6241-6252.

Jelinek, T., and Weber, M.J. 1993. Optimization of the resolution of phosphoamino acids by one-dimensional thin-layer electrophoresis. *Biotechniques* **15:** 628, 630.

Her, J.H., Lakhani, S., Zu, K., Vila, J., Dent, P., Sturgill, T.W., and Weber, M.J. 1993. Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation. *Biochemical Journal* **296:** 25-31.

Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M.J., and Sturgill, T.W. 1993. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3', 5'-monophosphate. *Science* **262:** 1065-9.

Rosen, L.B., Ginty, D.D., Weber, M.J., and Greenberg, M.E. 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12:** 1207-21.

Jelinek, T., Catling, A.D., Reu A., and Weber, M.J. 1994. RAS and RAF-1 form a signallin 2. *Molecular and Cellular Biology* **14:** 8212-18.

Peterson, J.E., T. Jelinek, M. Kaleko, K. Siddle, and M.J. Weber. 1994. Phosphorylation and Activation of the IGF-I Receptor in *src*-transformed Cells. *Journal of Biological Chemistry* **269**: 27315-21.

Catling, A.D., C.W.M. Reuter, M.E. Cox, S.J. Parsons, and M.J. Weber. 1994. Partial Purification of a Mitogen-activated Protein Kinase Kinase Activator from Bovine Brain. *Journal of Biological Chemistry* **269**: 30014-30021.

Reuter, C.W.M., A.D. Catling, T. Jelinek, and M.J. Weber. 1995. Biochemical Analysis of MEK Activation in NIH3T3 Fibroblasts: Identification of B-Raf and Other Activators. *Journal of Biological Chemistry* **270**: 7644-7655.

Catling, A.D., H.-J. Schaeffer, C.W.M. Reuter, G.R. Reddy, and M.J. Weber. 1995. A Proline-rich Sequence Unique to MEK1 and MEK2 is Required for Raf binding and regulates MEK function. *Mol. Cell. Biol.* **15**: 5214-5225.

Wright, J.D., C.W.M. Reuter, and M.J. Weber. 1995. An Incomplete Program of Cellular Tyrosine Phosphorylations Induced by Kinase-defective Epidermal Growth Factor Receptors. *Journal of Biological Chemistry* **270**: 12085-12093.

Dent, P., T. Jelinek, D.K. Morrison, M.J. Weber, and T.W. Sturgill. 1995. Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. *Science* **268**:1902-6.

Reuter, C.W., A.D. Catling, and M.J. Weber. 1995. Immune complex kinase for mitogen-activated protein kinase and MEK. *Meth. Enzymol.* **255**: 245-56.

Wright, J.D., C.W.M. Reuter, and M.J. Weber. 1996. Identification of sites on epidermal growth factor receptors which are phosphorylated by $pp60^{src}$. *Biochim. Biophys. Acta* **1312**: 85-93.

Jelinek, T., P. Dent, T.W. Sturgill, and M.J. Weber. 1996. Ras-Induced Activation of Raf-1 Is Dependent on Tyrosine Phosphorylation. *Mol. Cell. Biol.* **16**: 1027-1034.

Peterson, J.E., T. Jelinek, J.A. Shannon, and M.J. Weber. 1996. Phosphorylation Sites on the IGF-I Receptor due to Autophosphorylation or the Src Kinase. *J. Biol. Chem.* **271**: 31562-31571.

Kulik, G., A. Klippel, and M.J. Weber. 1997. Antiapoptotic Signalling by the Insulin-Like Growth Factor I Receptor, Phosphatidylinositol 3-Kinase, and Akt. *Mol. Cell. Biol.* **17**: 1595-1606.

Wei, S., A. M. Gamero, J. H. Liu, A. A. Daulton, N. I. Valkov, J. A. Trapani, A. C. Larner, M. J. Weber, and J. Y. Djeu. 1998. Control of Lytic Function by Mitogen-activated Protein Kinase/Extracellular Regulatory Kinase 2 (ERK2) in a Human Natural Killer Cell Line: Identification of Perforin and Granzyme B Mobilization by Functional ERK2. *J. Exp. Med.* **187**: 1753-1765.

Schaeffer, H.J., Catling, A.D., Eblen, S.T., Collier, L.S., Krauss, A. and Weber, M.J. 1998. MP1: A MEK Binding Partner that Enhances Enzymatic Activation of the MAP Kinase Cascade. *Science* **281**: 1668-1671.

Zecevic, M., Catling, A.D., Eblen, S.T., Renzi, L., Hittle, J.C., Yen, T.J., Gorbsky, G.G. and Weber, M.J. 1998. Active MAP Kinase in Mitosis: localization at kinetochores and association with the motor protein CENP-E. *J. Cell Biology* **142**: 1547-1558.

Kulik, G. and Weber, M.J. 1998. Akt-dependent and independent survival signaling pathways utilized by IGF-I. *Mol. Cell. Biol.* **18**: 6711-6718.

Chen T., R.W. Cho, P.J.S. Stork and M.J. Weber. 1999. Elevation of Cyclic Adenosine 3',5'-Monophosphate Potentiates Activation of Mitogen-activated Protein Kinase by Growth Factors in LNCaP Prostate Cancer Cells. *Cancer Research* **59**: 213-218.

Gioeli, D., J.W. Mandell, G.R. Petroni, H.F. Frierson and M.J. Weber. 1999. Activation of Mitogen-Activated Protein Kinase with prostate cancer progression. *Cancer Research* **59**: 279-284.

Gao, Z., Chen, T., Weber, M.J., and Linden, J. 1999. A_{2B} Adenosine and $P2Y_2$ Receptors Stimulate Mitogen-activated Protein Kinase in Human Embryonic Kidney-293 Cells. *J. Biol. Chem.* **247**: 5972-5980.

Schaeffer, H.J. and M.J. Weber. 1999. MAP Kinases: Specific Messages from Ubiquitous Messengers. *Mol. Cell. Biol.* **19**: 2435-2444.

Carson, J.P., G. Kulik and M.J. Weber. 1999. Anti-apoptotic Signaling in LNCaP Prostate Cancer Cells: A survival signaling pathway independent of PI3 kinase and Akt/PKB1. *Cancer Research.* **59**:1449-1453.

Slack, J.K., A.D. Catling, S.T. Eblen, M.J. Weber, and J. T. Parsons. 1999. c-Raf-mediated inhibition of epidermal growth-factor stimulated cell migration. *J. Biol. Chem.* **274**:27177-27184.

Nguyen, D.H.D., A.D. Catling[illegible]cer, A. Somlyo, M.J. Weber and S. L. Gonias. 1999. Myo[illegible]ity in an integrin-selective manner in urokinase-type plasmin[illegible]ell Biol.* **146**:149-164.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
George Kulik	Assistant Professor of Research

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Ukrainian Agricultural Academy, Ukraine	DVM	1986	Veterinary Medicine
Inst. Experimental Pathology, Ukraine	Ph.D.	1991	Biology
Imperial Cancer Research Fund, London	Postdoc.	1993-94	Signal Transduction

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

I have been working in the basic cancer research for 12 years. Initially I was studying signal transduction by EGF receptor and it role in carcinogenesis. For the last three years regulation of apoptosis by survival signaling became the focus of my research. I have described two survival signaling pathways one involves PI3K and Akt, another pathway independent on these enzymes. Since EGF receptor signaling and regulation of apoptosis directly connected and highly relevant to the pathogenesis of prostate cancer I believe that my experience will contribute in advancing our understanding of molecular mechanisms of this disease.

Recent research projects:
1. Role of PI3 Kinase and Akt in antiapoptotic signaling in fibroblasts and epithelial cells.
2. Novel survival signaling independent on PI3 Kinase and Akt in RIG fibroblasts.

Professional and Research Experience:
Research Assistant Professor since 1999
Research Associate with Michael Weber, University of Virginia, 1995-98
Post-doc with Peter Parker, Protein Phosphorylation Laboratory, ICRF, London, 1993-94
Research Scientist, Institute for Experimental Oncology, Kiev, Ukraine, 1992-93
Research Associate, Institute for Experimental Oncology, Kiev, Ukraine, 1991-92

Academic and Professional Honors:
First Prize for Research Work, Conference of Young Scientists, Kiev, 1989
Best Poster, 4th International Conference on Hormones and Cancer, Amsterdam, 1991
EMBO Fellowship, 1993
IUCC Fellowship, 1995

Publications:
Ivashchenko, Y., Goot, I., Garmanchuk, L., Kulik, G., Bykores, A. (1989). Changes in the number of epidermal growth factor receptors in hepatocytes during rat liver regeneration. *Cytologia* **11**: 453-460.

Ivashchenko, Y., Garmanchuk, L., Kulik, G. (1989). EGF-binding proteins in platelets and rat blood serum. *Reports of Acad. Sci. Ukrainian SSR* **B3**: 67-71.

Ivashchenko, Y., Garmanchuk, L., Philchenkov, A., Kulik, G., Kononenko, L., Bykores, A. (1990). Changes in the amount of epidermal growth factor and EGF-like polypeptides in the liver tissue of rats with hepatocarcinogenesis induced by N-diethylnitrosamine. *Experimental Oncology* **12**: 31-33.

Kulik, G., Ivashchenko, Y., Kononenko, L., Bykores, A. (1991). Influence of epidermal growth factor on the protein phosphorylation in normal and transformed hepatocytes. *Ukrainian Biochemical J.* **63**: 16-21.

Kulik, G., Ivashchenko, Y., Kononenko, L., Philchenkov, A., Bykores, A. (1991). Cytotoxic effect of anti-epidermal growth factor receptor antibodies on A-431 cells and primary culture of normal and transformed hepatocytes. *Experimental Oncology* **13**: 34-41.

Philchenkov, A., Ivashchenko, Y., Kulik, G. (1991). Purification of epidermal growth factor by hydrophobic chromatography. *Experimental Oncology* **13**: 71-74.

Ivashchenko, Y., Kulik, G., Philchenkov, A. (1991). Relationship between oligomerization state of epidermal growth factor receptor, its affinity and activation. *Bull. Exp. Biol. Med.*: 84-87.

RESOURCES

The laboratories of Dr. Myers and Weber are located on the second floor of the Jordan Hall research building. They are separated by less than 200 feet

Myers Laboratory

Laboratory. 1124 sq. ft. of modern laboratory space, plus shared facilities for radioisotope labeling, cold room and equipment rooms. All necessary equipment for tissue culture is present.

Clinical. We will interact with clinical prostate cancer activities through the Tissue Analysis Core laboratory, directed by Dr. Henry Frierson, who is the Pathologist primarily responsible for Prostate Cancer clinical specimens. Through him we have access to over 300 paraffin-embedded sections and 44 frozen radical prostatectomies.

Animal. A modern and very well-run vivarium is located in the basement of this building and has isolation areas for nude and other immunocompromised mouse colonies. Our mice are housed in one sterile room with HEPA filtered air, a laminar flow hood for handling the mice and performing surgery, and sterile blower racks for the mouse caging. In addition, we have an outstanding Hybridoma facility which has worked with us in the past to generate large quantities of monoclonal antibodies.

Computer. Our laboratory PCs are networked to the extensive University computer facilities and to the World Wide Web for data base searches. Medical Center Academic Computing also provides consultation services.

Office. Two 100 sq. ft. offices are adjacent to the laboratory space.

Other. The most important resource available is the support and expertise of my colleagues: Tom and Sally Parsons, Leland Chung and Dr. Michael Weber, with whom I interact daily. This is an incredibly rich environment for research on cellular regulation in cancer, with a wide variety of skills and an open, collaborative atmosphere.

Major Equipment. We possess or have ready access to all the major equipment needed for this work. In my own laboratory is an HPLC as well as centrifuges, biosafety cabinets, incubators, electrophoresis equipment, etc. The Biomolecular Core laboratories of the University of Virginia (located one floor below me) provide state-of-the art protein sequencing capabilities, including a Mass Spectrometry facility funded by the Keck Foundation. We have a Zeiss Axioskop fluorescence microscope equipped with a CCD camera and software for image acquisition capable of visualizing intracellular structures in living cells.

Weber Laboratory

Laboratory. 2000 sq ft of modern laboratory space, including facilities for radioisotope labeling, cell culture, protein chemistry and electrophoresis, cold room and equipment.

Clinical. We will interact with clinical prostate cancer activities through the Tissue Analysis Core laboratory, directed by Dr. Henry Frierson, who is the Pathologist primarily responsible for Prostate Cancer clinical specimens. Through him we have access to over 300 paraffin-embedded sections and 44 frozen radical prostatectomies.

Animal. A modern and very well run vivarium is located in the basement of this building and has isolation areas for nude mouse colonies. In addition, we have an outstanding Hybridoma facility which has worked with us in the past to generate monoclonal antibodies against Src and MAP Kinase and which is working with us now in the generation of phospho-specific monoclonals.

Computer. Our laboratory PCs are networked to the extensive University computer facilities and to the World Wide Web for data base searches. Medical Center Academic Computing also provides consultation services on sequence analysis, structure determinatio

Office. My office is windowless and tiny, but there are nearby conference rooms which have windows and are large enough for research-group meetings.

Other. The most important resource available is the support and expertise of my colleagues: Tom and Sally Parsons, Leland Chung and Charles E. Myers, with whom I interact daily. In addition, I have a long-standing productive relationship with Dr. Donald Hunt of our Chemistry Department who is a leading expert on protein sequencing by mass spectrometry. This is an incredibly rich environment for research on cellular regulation in cancer, with a wide variety of skills and an open, collaborative atmosphere.

Major Equipment. We possess or have ready access to all the major equipment needed for this work. In my own laboratory are three BioRad FPLC instruments and an HPLC as well as centrifuges, biosafety cabinets, incubators, etc. The Program Project I participate in (Oncogenes and Mitogens: Intracellular Mechanisms/CA40042) has purchased equipment for immunofluorescence, microinjection and video microscopy, which is available to me in an adjacent laboratory. This includes a Zeiss IM35 with fluorescence attachments, heated stage, CCD camera, micromanipulator, and a Brinkmann microinjector. In addition, we have a standard Leitz Fluorescence microscope. However, these are very heavily used and not up-to-date. The Biomolecular Core laboratories of the University of Virginia (located one floor below me) provide state-of-the art protein sequencing capabilities, including a Mass Spectrometry facility funded by the Keck Foundation.

BACKGROUND AND SIGNIFICANCE

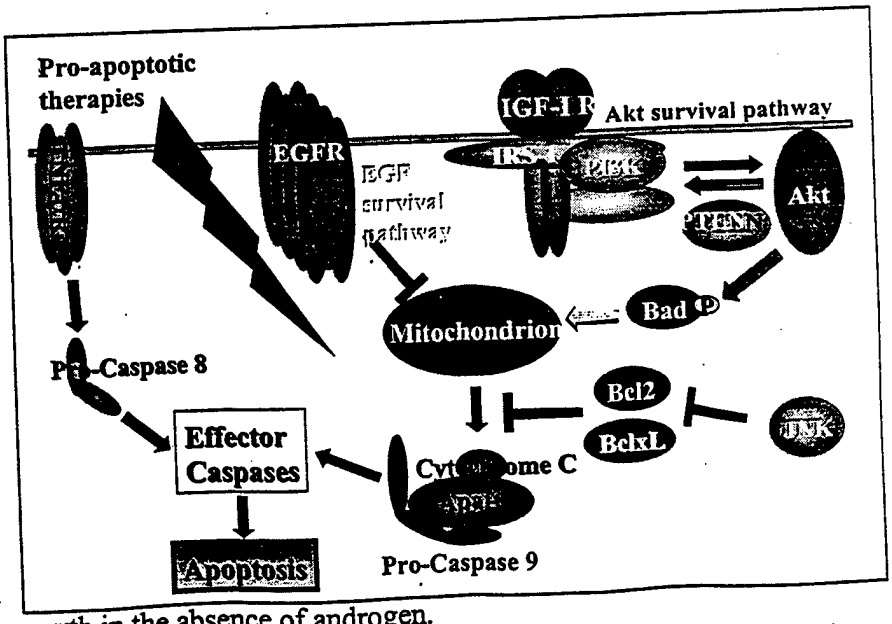
Success of Androgen-Withdrawal, Chemotherapy and Radiation is Limited in Prostate Cancer

Androgen-withdrawal plays a major role in the treatment of prostate cancer. This method of treatment owes much of its popularity to the fact that even in men with advanced prostate cancer, up to 80% will obtain relief of symptoms such as bone pain following hormonal therapy. On the other hand, complete remissions are seen in less than 10% of the men with widespread metastatic disease[1] Pathologic examination shows that -- in contrast to the normal organ -- only a minority of tumor cells typically enter apoptosis following androgen ablation, while a larger proportion of the tumor cells undergo growth arrest[2]. As a result, large numbers of cancer cells survive androgen ablation [3] and hormone-resistance inevitably develops. While less well studied, it appears that a similar process limits the success of chemotherapy and radiation therapy in this disease: partial responses are now a common result from combination chemotherapy but median duration of response is generally well under one year.

There has been enormous recent progress in our understanding of apoptosis and its regulation. As a result of these advances, we now understand some of the major factors that limit initial response to treatment and shorten its duration. The central problem is that several powerful survival factors available to prostate cancer cells and these other pathways limit the response of this cancer not only to androgen withdrawal, but also to radiation and chemotherapy.

Mechanisms of Prostate Cancer Cell Survival

An approach to improving prostate cancer treatment is to identify and systematically block the molecular mechanisms by which prostate cancer cells survive treatment. Response to androgen ablation has been studied most extensively and is good model of the molecular basis of treatment resistance. It is already apparent that prostate cancer cells have multiple, redundant mechanisms available that can foster their survival and growth in the absence of androgen.



Bcl-2 family members. One of the first survival mechanisms detected and studied in prostate cancer involves regulation of Bcl-2. Overexpression of Bcl-2 correlates with advanced disease and ectopic expression is sufficient to provide a substantial growth advantage to the cells and render them resistant to toxic insults [4]. Expression of two other members of the Bcl-2 family, Bcl-x, and mcl-1, also correlated with high pathologic grade and in metastatic disease as compared to non-metastatic cancer and PIN[5]. Prostate cancer cells respond to androgen ablation by increased expression of [illegible] androgen independent cells often exhibit increased

expression of Bcl-2[2, 6, 7]. Finally, in experimental models, Bcl-2 overexpression speeds the development of hormone-independent disease [4, 8]. At least two approaches to neutralizing the anti-apoptotic effects of Bcl-2 are currently being investigated in several laboratories. First, Bcl-2 antisense slows the development of hormone-independent disease and currently is in clinical trial[9, 10]. Second, Bcl-2 can be inhibited by phosphorylation which is triggered by certain microtubule stabilizing drugs (e.g. taxotere) via a cell-cycle activated signaling pathway apparently dependent on JNK (see Figure)[11].

Prostate cancer cells also express Bcl-xL and in LNCaP this appears to be the dominant member of the Bcl-2 family. Bcl-xL is also phosphorylated and inhibited following addition of taxanes[12]. Additionally, expression of Bcl-xl is suppressed by the taxanes [12]. Bcl-xL can also be suppressed by antisense constructs [13]. We propose to make use of both these approaches to reduce the resistance of prostate cancer cells to apoptosis.

In summary, it seems clear that Bcl-2 overexpression alone is sufficient for prostate cancer cells to survive androgen withdrawal. It is also clear that it is not the only mechanism that can do this.

PI3 Kinase and Akt. Another well-characterized survival pathway involves activation of the serine/threonine kinase Akt. This enzyme is activated by the products of PI3 Kinase, an enzyme which in turn is activated in response to various "survival factors" such as IGF-I. Integrin engagement also provides a survival effect, and the current evidence indicates that much if not all of this effect is through the PI3 Kinase/Akt pathway. Akt is able to inhibit cell death by phosphorylation and inactivation of multiple targets, including: Bad, a proapoptotic member of the Bcl-2 family; FKHRL1, a transcription factor that controls expression of the gene for Fas-ligand; and caspase 9 [14]. The products of PI3 Kinase are negatively regulated by the lipid phosphatase PTEN. However, PTEN deletion correlates very well with high Gleason grade and advanced stage, and in these cells Akt is constitutively active and this survival pathway is always operative[15]. At least in the LNCaP cell line, transfection with functional PTEN induced apoptosis that could be blocked by overexpression of Bcl-2 [16], indicating that either the presence of a PTEN deletion or the overexpression of Bcl-2 was sufficient for survival.

Because of its central role in survival signaling, several pharmaceutical companies are developing Akt inhibitors. In addition there are several tools available to inhibit PI3 Kinase and thus reduce Akt activity, including Wortmannin and LY294002. In LNCaP cells, these PI3 kinase inhibitors cause apoptosis under serum-free conditions. While these inhibitors have not been tested in vivo against prostate cancer, wortmannin has been given orally to mice bearing a range of non-prostate tumor xenografts and shows only modest antitumor efficacy [17].

However, we recently have shown that in prostate cancer cells there are multiple survival pathways and that several if not all will probably have to be inhibited if androgen ablation or other therapies are to be maximally pro-apoptotic [18]. Inhibition of Akt by blocking PI3 Kinase induces apoptosis in LNCaP cells only if the cells are deprived of all other "survival factors" including androgen, serum or EGF. Thus we predict that androgen ablation or other treatments will induce extensive apoptosis of prostate cancer cells only if these alternative pathways are also inhibited. These pathways, and the tools for intercepting them, are described now.

Epidermal Growth Factor Receptor Family. There are several lines of evidence that point to a role for the EGF Receptor in the development of hormone-resistant prostate cancer. Normal prostatic tissue components produce amphiregulin and heparin-binding Epidermal Growth Factor, two EGFR ligands [20]. Co-expression of EGFR and TGF-alpha is not characteristically observed in normal prostate epithelial cells nor is it common in hormone-sensitive, pathologically low or moderate grade tumors. However, co-expression of EGFR and

TGF-α is common in tumors of high pathologic grade and after the development of hormone-refractory disease [19].

In prostate cancer cells, EGFR activation has been documented to enhance tumor cell growth and invasiveness [21]. EGFR activation has been shown to inhibit apoptosis in a wide range of cell lines. While EGFR activation can promote cell survival through activation of PI3 Kinase in some cell lineages that massively overexpress the EGFR, we have recently shown that in prostate cancer LNCaP cells EGFR promotes survival by a mechanism that is independent both of PI3 Kinase and Akt [18].

A related receptor, HER2, is also expressed in prostate cancer cells and expression of this receptor is associated with a poor prognosis [22-24]. HER2 does not have a known ligand, but can form complexes with other EGFR family receptors, leading to enhanced signaling by the HER2 partner.

Additionally, in prostate cancer cells, the IL-6 receptor appears to require the presence of HER2 in order to activate MAP kinase[24]. In advanced prostate cancer IL-6 appears to act in an autocrine manner to promote growth[25]. Thus, an inhibitor of HER2 might be expected to simultaneously interfere with IL-6 autocrine signaling.

Because the EGFR and its relatives play a key role in epithelial tumors, a wide range of drug candidates are under development by the pharmaceutical industry. One tool for interdicting EGFR signaling is the anti-EGFR antibody, C225 [26, 27]. This agent is currently in Phase 2 clinical trials for prostate cancer and other epithelial cancers, at this and other institutions. Across a wide range of cell lineages, including prostate cancer, treatment with C225 sensitizes tumor cells to the cytotoxic effects of chemotherapy and gamma radiation. Although it is not known how C225 (and the HER2 antibody, Herceptin) potentiate cell killing, there is evidence to suggest that they do so by potentiating drug-induced apoptosis, a result consistent with our hypothesis. For a study such as ours, in which we hope to obtain mechanistic as well as pre-clinical information, the lack of clear mechanisms for C225 is a disadvantage. However, its advanced stage of clinical development and lack of substantial toxicity are advantageous.

There also are promising and potent small molecule inhibitors of the EGFR. Notable is Iressa (ZD1839) from Astra Zeneca, which is now in clinical trials, and PD183805, from Parke-Davis/Warner-Lambert. The latter compound is an irreversible inhibitor of the EGFR family, and is active in low nanomolar concentrations. We are in the process of gaining access to these drugs.

Neuropeptide receptors. Human prostate cancers commonly contain neuroendocrine cells that have been shown to produce neuropeptides, including serotonin, bombesin, PTH-RP, and calcitonin. Several of these neuropeptides stimulate the growth of prostate cancer cells in tissue culture. In prostate cancer, androgen-withdrawal triggers an increase in the number of neuroendocrine cells and the number of neuroendocrine cells has been reported to be higher in hormone-refractory tumors [28-30]. In patients undergoing androgen withdrawal, elevation of serum chromogranin levels is strongly associated with subsequent appearance of hormone refractory disease[31].

The neuropeptides that increase the proliferation of prostate cancer cells act by increasing intracellular cAMP levels, leading to the activation of PKA [32]. In many cell types, activation of PKA is associated with growth arrest and differentiation. In prostate cancer cells, activation of PKA inhibits apoptosis. We have recently shown that epinephrine and other neuroendocrine factors that stimulate prostate growth act synergistically with EGFR to activate MAP Kinase, which in some cell lineages and conditions is anti-apoptotic [32]. Activation of MAP Kinase by PKA signals through the small GTP'ase Rap-1 and B-Raf [33]. We have shown that prostate

cancer cell lines, especially LNCaP, express B-Raf at levels approaching that seen in neural cells [32]. PKA also phosphorylates the pro-apoptotic Bad protein on S112, inactivating it (comparable to Akt, which phosphorylates on S136). Thus, PKA is able to inhibit apoptosis. potentially, by at least two mechanisms, one involving MAP Kinase activation and the other by phosphorylation of Bad [34].

While inhibitors of PKA exist and are useful experimental tools, PKA is involved in many biological processes including growth arrest and differentiation in many cell lineages For example, 8-Cl-cAMP, a PKA inhibitor currently undergoing clinical evaluation, causes hypercalcemia. For our *in vitro* mechanistic studies, we will use the potent, selective PKA inhibitor, H89 [35] as well as 8-Cl-cAMP to test whether PKA-dependent survival signaling plays an important role in limiting the response to androgen ablation.

On the other hand, B-Raf appears to have a much more restricted role. We have gained access to a B-Raf antisense construct developed by ISIS Pharmaceuticals and plan to use this to test whether PKA-dependent survival signaling can also be blocked by this more selective agent.

Pro-apoptotic signaling

FAS and FAS-L should be attractive targets for induction of pro-apoptotic signaling in prostate cancer. Fas likely plays a role in involution of the prostate after androgen ablation as this does not occur in *lpr* mice which are deficient in functional FAS. Androgen withdrawal results in up to a 5-fold increased expression of FAS in prostate cells. This is associated with binding of the adapter protein FADD [38, 40]. Expression of FAS and FAS-L are the rule in human prostate cancer cell lines and in human tumor specimens obtained at radical prostatectomy. In tissue culture, the common human prostate cancer cell lines constitutively release soluble FAS-L (sFAS-L) into the media in amounts sufficient to kill a sensitive indicator cell line. However, apoptotic signaling in the prostate cancer cells is blocked down stream of FADD. The factors that hinder FAS-mediated cell death in human prostate cancer have not been defined.

We have recently reexamined the biology of FAS and FAS-L in LNCaP and PC3 prostate cancer cell lines (Preliminary Data). We found that both cell lines have large intracellular stores of both proteins with relatively little to be found on the cell membranes. The amount released into the media as s-FAS-L is also minuscule compared to the intracellular stores. For example, S-FAS-L only kills the human prostate cancer cell lines when it is isolated from prostate cancer cell conditioned media, concentrated and re-presented at higher concentrations [36]. The implication of these findings is that part of the resistance of prostate cancer cells to FAS-induced apoptosis may result from the fact that most of the FAS and FAS-L are sequestered intracellularly rather than on the cell surface.

We had previously shown that the eicosanoid, 5-HETE, is a potent survival factor for both LNCaP and PC3 cells[41]. When production of this eicosanoid is blocked, apoptosis proceeds rapidly and most of the cells are dead within eight hours. When we examined the role of FAS and FAS-L in this system, we found that cell death was preceded by translocation of both FAS and FAS-L to the cell membrane within 30 minutes after 5-HETE production was blocked. At that time point, mFAS-L and FAS had clustered at the cell membrane and caspase 8 activation had commenced. These findings suggest that 5-HETE functions as a survival factor by preventing the translocation of FAS and FAS-L to the cell surface. Prostate cancer cells synthesize 5-HETE from arachidonic acid and we have found that 10 μM arachidonic acid bound to serum albumin provides optimal conditions for 5-HETE formation and the survival of LNCaP cells.

Radiation exposure and exposure to cytotoxic drugs each interact with FAS to promote tumor cell death [42, 43]. Since prostate cancer cells do not array FAS or FAS-L on their cell surface under routine tissue culture conditions, it is possible that translocation of these molecules to the cell surface may play a role in the killing of prostate cancer cells by wide range of agents. Furthermore, approaches, such as inhibition of 5-HETE formation, that stimulate massive translocation of FAS and FAS-L to the cell surface may well enhance the effectiveness of other therapies (see Preliminary Data section).

Taken together, these findings suggest that tumor cell kill might be enhanced by combining proapoptotic therapies that activate various death receptors with inhibition of survival signaling pathways.

Treatment Failure In Patients Is Likely to Be Complex

There seems to be no *a priori* reason why prostate cancer cells can not possess multiple survival mechanisms simultaneously. The human hormone-responsive cell line, LNCaP, is an example of this problem. The growth of LNCaP *in vitro* slows when androgen is absent, but the cells do not undergo apoptosis unless all other survival factors are withdrawn and Akt is inhibited [18]. *In vivo*, androgen ablation is associated with transient arrest of tumor growth followed by the development of androgen-independent disease. LNCaP owes its survival to multiple mechanisms. The androgen receptor in this cell line is mutated so that it will respond to estrogens, progestins and the antiandrogen, hydroxyflutamide as well as it does to testosterone or dihydrotestosterone. LNCaP lacks the phosphatase, PTEN [16], leading to constitutive activation of the Akt survival pathway. LNCaP produces TGF-alpha and expresses EGFR, leading perhaps to some constitutive activation of EGFR [21]. Without some sensitizing event, LNCaP is relatively resistant to FAS-L and TNF-alpha. *In vivo*, LNCaP cells develop increased numbers of neuroendocrine cells when subjected to androgen withdrawal[28]. All of these factors render LNCaP quite resistant to apoptosis and make it a useful model of therapy resistant human prostate cancer.

Making the Response to Treatment More Complete

Among clinicians involved in cancer treatment, there are several standard approaches to enhancing a therapy that is partially effective. One involves intensification of that treatment in an attempt to improve the response. Unfortunately, efforts to intensify androgen ablation or chemotherapy have led to only minor changes in the effectiveness of treatment for metastatic prostate cancer. Another approach is to combine partially effective treatments, hoping that the results will be synergistic or at least additive. An example of this approach would be the combination of external beam radiation therapy with androgen withdrawal. This approach appears to have met with some success as the combination lessens the risk of prostate cancer recurrence in the prostate, as well as at distant sites. Similar thinking has also led to a series of clinical trials in which androgen ablation and chemotherapy have been combined. For example, we have recently reported a Phase II clinical trial combining androgen ablation with suramin[44].

The current proposal was stimulated by a recent paper by one of us (MJW) that examined the role of the Akt survival pathway in LNCaP. In this study, we found that the PI3 Kinase inhibitors, wortmannin and LY294002 triggered apoptosis in LNCaP cells if they were grown under serum-free conditions. However, the addition of androgen, EGF or serum allowed these prostate cancer cells to survive. These findings suggest to us that any approach based on blockade of only one survival mechanism is likely to meet with limited success. Other lines of evidence support this concern. The anti-EGFR antibody, C225, has already been tested against

prostate cancer in animal models and in patients with prostate cancer. While C225 can slow the growth of prostate cancer, this agent does not cause complete remissions. The PI3 Kinase inhibitor, wortmannin, has been tested against human tumor xenografts in immunocompromised mice and complete remissions were not seen. These considerations have led us to the hypothesis that: ***Treatment will routinely cause complete remissions only if it involves simultaneous blockade of the multiple mechanisms responsible for prostate cancer cell survival.***

The focus here will be on survival signaling pathways that act post-transcriptionally and thus we will not deal with p53 and NFκB. The post-transcriptional survival regulation is more amenable to experimental manipulation. In addition, our work has already shown that these signaling pathways are epistatic to (downstream from) the p53 status or expression of NFκB and thus their manipulation is sufficient to alter the sensitivity of cells to apoptosis.. This makes sense since the transcriptional regulation acts at least in part by changing the concentrations of the same components (e.g. Bcl-2, FAS) that we propose to alter directly.

Combined Survival Pathway Inhibition Is Timely

While our knowledge of the survival pathways that limit response to treatment is probably incomplete, there is value in understanding the relative contributions of the currently known pathways. Reagents that inhibit individual survival pathways in prostate cancer are on the market (Herceptin and the taxanes), in advanced clinical trials (C225), early clinical trials (antisense Bcl-2) or are targets of drug development by major pharmaceutical firms (PD183805 for Her family members, anti-sense B-Raf). Clinical trials testing combinations of these agents will soon be possible. At present, there are no theoretical or practical guidelines for how to design clinical trials testing inhibitors of multiple survival pathways. The goal of this application is to determine how best to combine the reagents we currently have so as to maximize the response to treatment.

The studies outlined in this proposal are clearly of translational intent and may result in a clinical trial. However, they will also serve a number of basic research purposes. While work has proceeded rapidly on the identification and function of individual survival pathways in well-defined *in vitro* models, little is known about how these pathways interact *in vivo* to support the survival of cancers such as carcinoma of the prostate. The experiments proposed in this grant application will provide a much better understanding of the relative contribution of these individual pathways.

Perhaps the most interesting scientific result would be the discovery that inhibition of all of the survival pathways outlined above do not result in a complete response. The cancer cells that emerge following this treatment may manifest previously undiscovered anti-apoptosis mechanisms. Should this occur, the investigators on this project are well equipped to investigate new apoptosis pathways.

PRELIMINARY RESULTS

Signal transduction regulates the sensitivity of cells to apoptosis.

Weber and Kulik were among the first to show that IGF-I functions as a survival factor by activating PI3 Kinase and Akt. They found that activation of PI3 Kinase or Akt was necessary for IGF-I to protect cells from apoptosis and that mutationally activated Akt was sufficient to protect cells from UV-induced apoptosis in the absence of IGF-I [53]. They also found that EGF did not function as a survival factor in fibroblasts although it was an effective mitogen and activator of MAP Kinase.

With the participation of a graduate student, Jon Carson, they then turned their attention to prostate cancer cells, focusing predominantly on LNCaP as a model system. These cells proved to be relatively resistant to apoptosis induced by UV and other agents, suggesting that they had up-regulated survival signaling pathways. In these cells PTEN is deleted and as predicted Akt was constitutively activated [18]. However Akt could be inactivated by inhibiting PI3 Kinase with wortmannin or LY294002 (Fig. 1). When PI3 Kinase and Akt were inhibited the cells rapidly underwent apoptosis if they were kept in serum-free medium (Fig.2). However, apoptosis was prevented if the cells were kept in androgen, serum or EGF (Fig. 3). Protection by androgen required new RNA and protein synthesis and 24 hours of exposure (data not shown), and we suspect that there will be multiple effectors of the steroid. However protection by EGF occurred within minutes and did not require new RNA or protein synthesis. Thus the EGF-induced resistance to apoptosis was due to a signal transduction pathway operating post-transcriptionally, post-translationally and rapidly. Importantly for purposes of this application, the EGF-induced survival signals did not involve activation of Akt (Fig. 4) or phosphorylation of Bad (Fig. 5). This demonstrates the existence of a novel and lineage-restricted survival signaling pathway independent of and redundant to the PI3 Kinase/Akt pathway.

In addition, increasing levels of cAMP also induced an Akt-independent survival pathway, and preliminary results indicate that Bad is also not involved (data not shown). EGF did not elevate cAMP in LNCaP cells, and inhibition of PKA with

Figure 1.

Inhibition of constitutive Akt/PKB phosphorylation in LNCaP and stimulated Akt/PKB phosphorylation in DU145

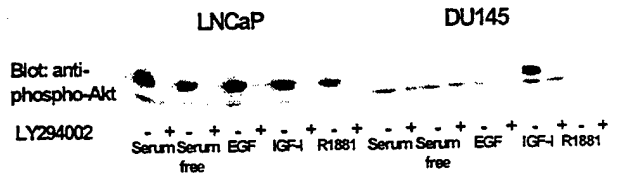


Figure 2.

Time course of LY294002-mediated apoptosis in LNCaP in serum free media

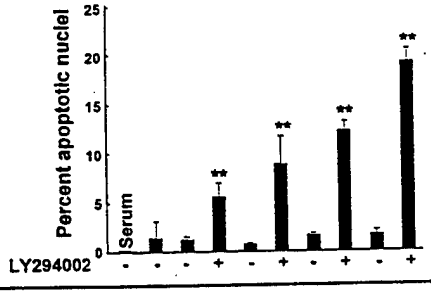
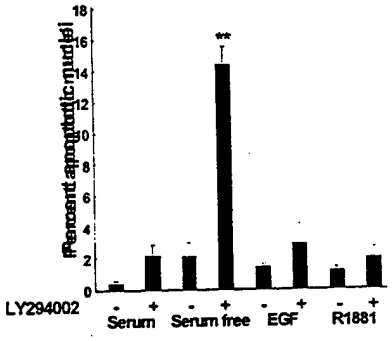


Figure 3.

Protection from LY294002-mediated apoptosis by 10% FCS, EGF and R1881 in LNCaP cells



H89 did not block the effectiveness of EGF. Thus we believe there is yet another survival pathway, dependent on cAMP and independent of PI3 Kinase, Akt and the EGF pathway.

The therapeutic implications of these findings are as follows: ***prostate cancer cells can become resistant to pro-apoptotic therapies by exposure to androgen and by at least three separate signal transduction-based mechanisms:***

1. Inactivation of PTEN by deletion, mutation or transcriptional silencing.
2. Upregulation of EGF signaling through autocrine and paracrine loops, whose existence in advanced cancer is well-established.
3. Increased levels of cAMP, that would be expected to occur in response to neuropeptides produced by the "neuroendocrine" cells that often are seen in prostate cancers.

Figure 4.

EGF does not activate Akt in the presence of LY294002

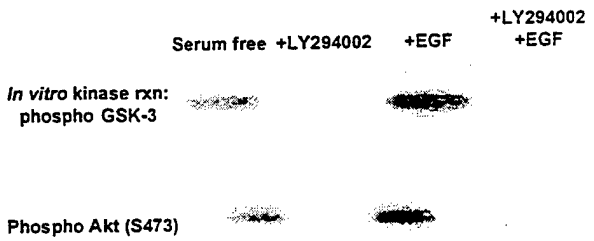
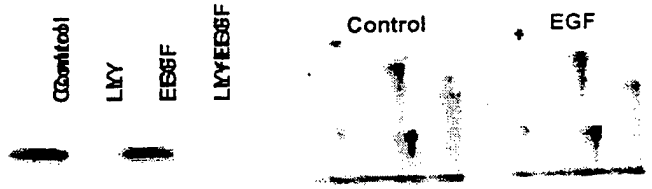


Figure 5.

EGF does not stimulate phosphorylation of Bad



Bad immunoprecipitated from LNCaP cells loaded with ^{32}P for 8 hours

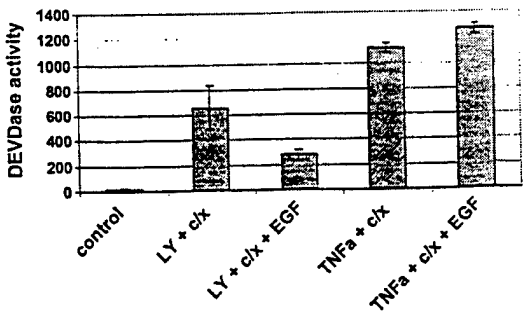
Tryptic phosphopeptide maps of Bad

Thus, if one wishes to sensitize these cells to pro-apoptotic treatments it will be necessary not only to remove androgen and inactivate Akt (which is a target for numerous pharmaceutical companies) but also to inactivate these other pathways. Although our understanding of the molecular mechanisms by which these pathways operate is still incomplete, it is already evident that pharmacological tools exist for inhibiting them. Thus, the time is ripe for assessing the ability of combinations of these agents to increase the effectiveness of therapies for prostate cancer.

A complementary approach to enhancing the ability of therapies to induce apoptosis in prostate cancer is to seek therapies whose mechanisms for inducing apoptosis might bypass the known survival pathways. The death pathway initiated by cytokines such as TNF-α and Fas Ligand can bypass the requirement for cytochrome c and caspase 9 (See Figure in Background and Significance) and thus conceivably could bypass the survival effects of Akt and perhaps EGF as well. To test this, LNCaP cells were treated with TNF-α

Figure 6.

EGF cannot protect from TNF-α-induced apoptosis



with or without EGF, but without inhibition of the PI3 Kinase/Akt survival pathway. The data in Fig. 6 demonstrate that TNF-α can trigger apoptosis even when the known survival pathways are active. This is not due to an effect of TNF-α on the ability of EGF to signal or on the activity of Akt (data not shown).

The strategy proposed here is to ablate singly and in combination the known survival pathways to determine whether the cells will display increased sensitivity to pro-apoptotic agents. A particular focus will be on identifying inhibitors and cytotoxic agents that act on convergence points of these pathways (e.g. taxol and antisense Bcl-2 or Bcl-xL) and on identifying pro-apoptotic agents that will bypass the survival signals (e.g. Fas-L).

Arachidonic Acid Promotes the Survival and Growth of Prostate Cancer Cells

Dietary intake of the omega 6 fatty acid, linoleic acid, was shown by Heston, et al to promote the growth of LNCaP in a xenograft model. In mice, linoleic acid is rapidly converted to arachidonic acid. For this reason, we assessed the impact of arachidonic acid on the growth and survival of PC3 and LNCaP cell lines. Under serum-free conditions, albumin-bound arachidonic acid caused greater than a 2-fold increase in the growth of LNCaP cells [41]. The optimal concentration for growth was 10 μM.

Arachidonic acid undergoes conversion to a series of eicosanoids. We inhibited the formation of various eicosanoids and established that it was the conversion of this fatty acid to 5-HETE by 5-lipoxygenase that was responsible for the proliferation induced by 5-HETE.

During these experiments, we noted that when 5-lipoxygenase was fully inhibited, both LNCaP and PC3 cells undergo an apoptotic death within 8 hours and that the cells could be rescued by 5-HETE [41]. We have subsequently investigated the mechanism by which the inhibition of 5-lipoxygenase causes apoptosis. We found that apoptosis in this case was the result of activation of FAS by a novel mechanism. Both LNCaP and PC3 cells express FAS and FAS ligand (FAS-L). Using immunofluorescence, we demonstrated that a large proportion of both FAS (not shown) and FAS-L were sequestered intracellularly and relatively little of either molecule could be found on the cell surface (Figure 7).

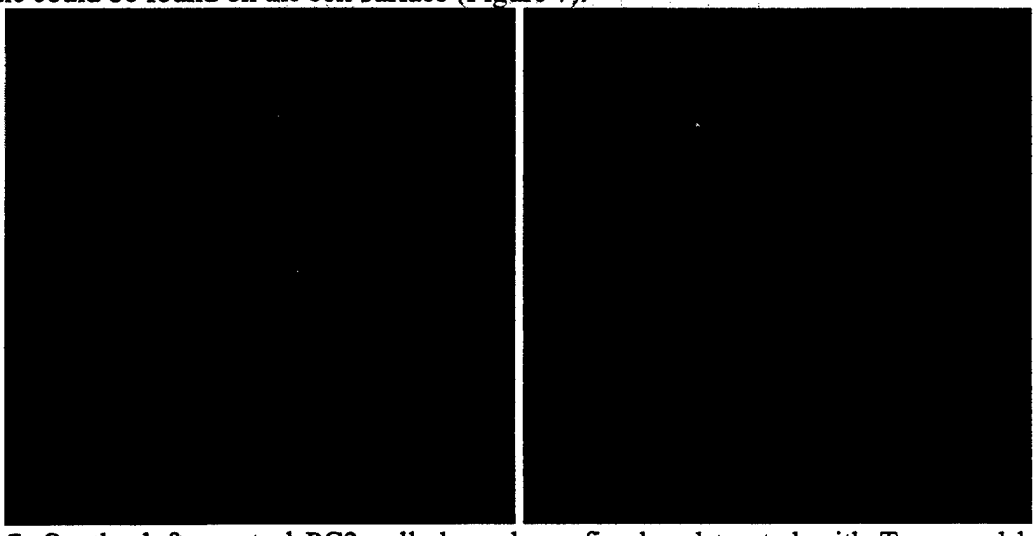


Figure 7. On the left, control PC3 cells have been fixed and treated with Texas red-labeled polyclonal anti-FAS-L antibody. This image shows large intracellular stores of FAS-L. On the right, live control PC3 cells have been exposed to fluorescein-labeled polyclonal anti-FAS-L

antibody, showing comparatively little FAS-L on the cell surface. Similar results were obtained with LNCaP cells.

After the addition of the 5-lipoxygenase inhibitor, Mk 886, an increase in FAS-L was apparent at 15 minutes and reached a maximum by 30 minutes. By 30 minutes FAS was also apparent on the cell surface and this reached a peak at 1.25 hours (Figure 8).

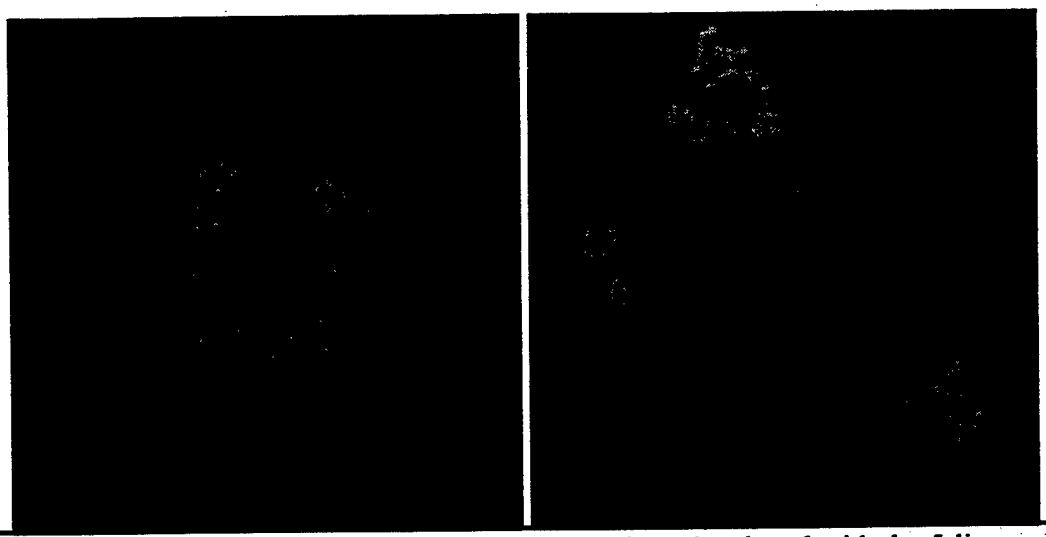


Figure 8. In the panel on the left, living PC3 cells have been incubated with the 5-lipoxygenase inhibitor, MK886, for thirty minutes. The cells were then exposed to fluorescein-labeled anti FAS-L antibody. This image shows FAS-L on the cell surface gathered into clusters. In the panel on the right, live PC3 cells were incubated with MK886 for 1.25 hours. The cells were then exposed to fluorescein-labeled anti-FAS antibody. This image shows a dramatic increase in FAS at the cell surface compared to control (not shown). Similar results were obtained with LNCaP cells.

This was associated with recruitment of both FADD (Figure 9) and procaspase 8 (not shown) to cell surface FAS. This was then followed by rapid activation of caspase 8 followed by caspase 3 (not shown). Neutralizing antibodies to FAS and FAS-L were sufficient to protect the cells from apoptosis. ell death was not prevented by the addition of EGF. In the presence of serum, the concentration of MK886 required to induce death increased 10 fold, but otherwise proceeded as rapidly as in the absence of serum.

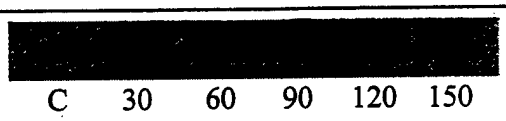


Figure 9. FADD association with FAS in PC3 cells. The times listed are from the point of MK886 addition in minutes. In this experiment, the Death-inducing Signaling Complex (DISC) was immunoprecipitated using monoclonal anti-FAS antibody (APO-1-3, Kamiya). FADD was visualized using monoclonal anti-FADD antibody. Identical results were obtained with LNCaP cells.

LNCaP cells lack functional PTEN while PC3 expresses wild type PTEN, yet cell death proceeded with equal rapidity in both cell lines. In LNCaP cells, Akt is constitutively activated. The phosphorylation of Akt did not change during apoptosis induced by MK886 in LNCaP (not shown), confirming that FAS-associated apoptosis in this case was independent of Akt. **The therapeutic implications of these findings are that this pro-apoptotic stimulus effectively bypasses both EGF- and PTEN-dependent survival pathways.**

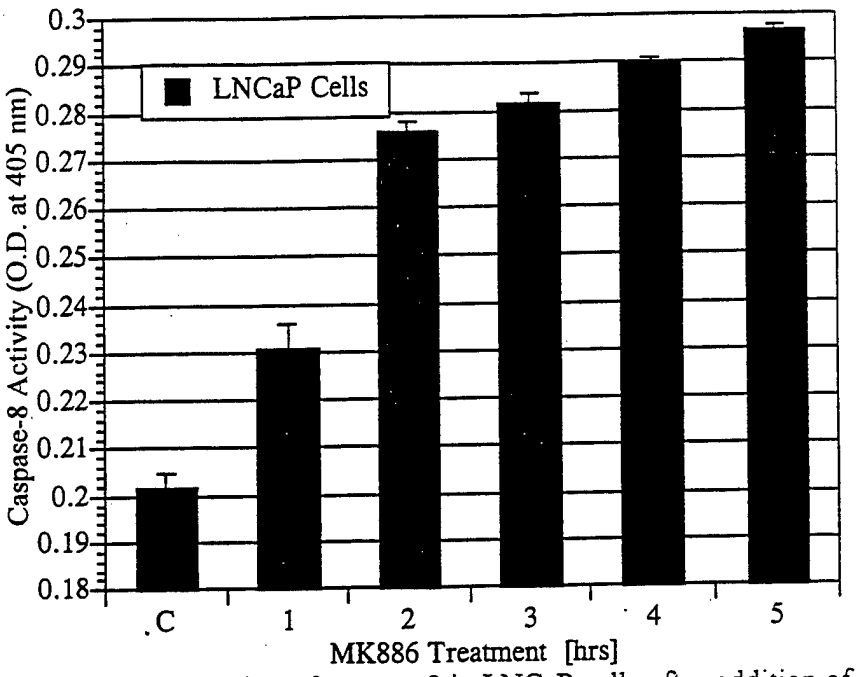


Figure 10. Activation of caspase 8 in LNCaP cells after addition of MK886. Caspase 8 activity in cell lysate was measured using caspase assay kit from BIOMOL.

Specific AIM I.

Utilize cell culture models to determine which survival signaling pathways need to be inactivated to enhance the effectiveness of pro-apoptotic therapies.

Rationale

It is becoming increasingly clear that resistance of prostate cancer cells to treatment is determined by several survival mechanisms, each of which may be sufficient to protect cells from apoptosis. Thus, successful treatment is likely to require inhibition of multiple anti-apoptotic pathways. We expect that inhibition of different survival signaling pathways will synergistically enhance the effectiveness of cytotoxic therapies.

Tissue culture model.

We have chosen initially to focus on the LNCaP cell line because its behavior as a xenograft resembles advanced prostate cancer in that it only marginally responds to androgen withdrawal and other therapies. Compared to other cultured cell lines we have tested, LNCaP cells are relatively insensitive to the drugs used in cancer therapy. One factor that supports the survival of LNCaP is that PTEN is deleted in these cells and c-Akt is constitutively active even under serum free conditions. PTEN is deleted or inactive in perhaps a majority of advanced prostate cancers, and thus LNCaP is representative of this group of intrinsically therapy-resistant tumors.

For most patients the site where hormone-resistant disease first becomes clinically apparent is the bone, suggesting that the bone environment might support survival and subsequent growth of this cancer in the face of androgen withdrawal. Dr. Leland Chung, one of the co-investigators on this SPORE application, has published extensively on the interaction between prostate cancer and stromal cells. He has shown that stromal cell lines derived from the prostate or bone marrow stimulate the growth of LNCaP in tissue culture and *in vivo*. With the guidance of Dr. Chung, we plan to use this mixed cell culture system in our experiments.

To create a situation in which the known survival pathways are activated, LNCaP will be grown in T-medium supplemented with serum, EGF and forskolin. The drug combinations that appear as most promising under these conditions will subsequently be retested in coculture with the MS osteoblastic cell line. This approach will allow us to do "in vitro" screening under conditions that more nearly match the environment in which bone metastases form than is the case for standard tissue culture conditions.

Optimizing the combination of drugs.

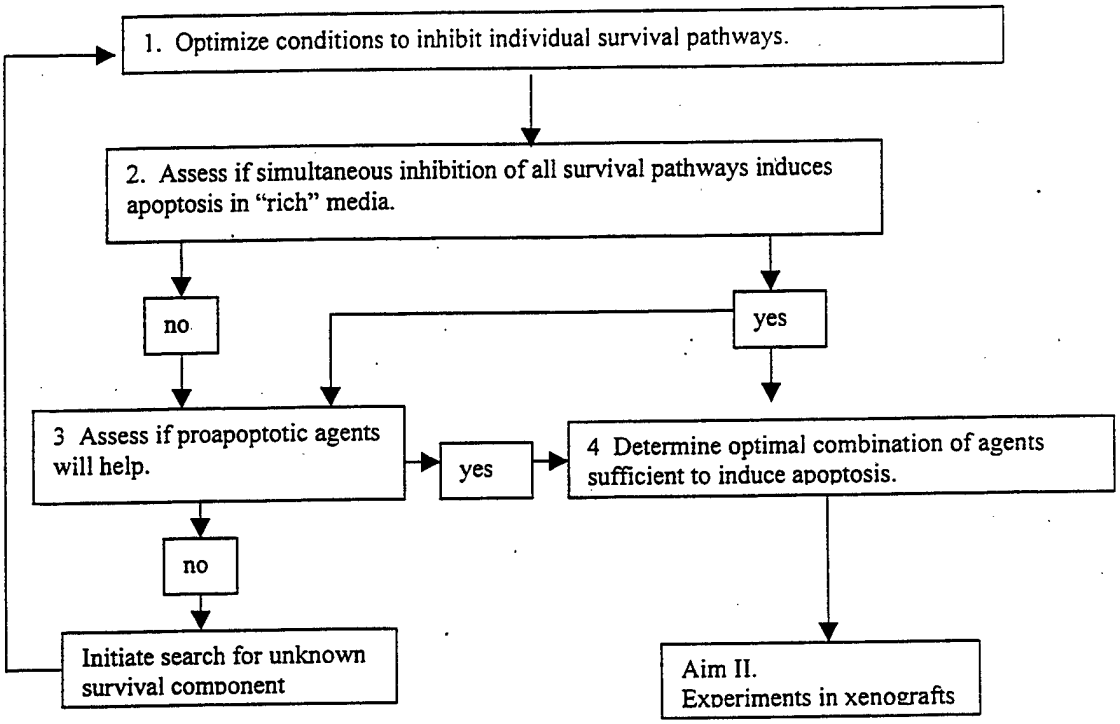
Anyone seeking to block the multiple signaling pathways that have been implicated in prostate cancer cell survival must confront serious logistical problems. By our count, there are at least five anti-apoptotic targets: (1) PI3 kinase/AKT; (2) HER family of receptor tyrosine kinases; (3) PKA/B-Raf; (4) Bcl-xL; (5) Androgen Receptor. In addition, we have selected two pro-apoptotic treatments that have shown promise *in vitro* or clinically: (1) activation of FAS/FAS-L and (2) Taxol.

Since cancer is characterized not so much by the appearance of new phenotypes as by the appearance of new combinations of phenotypes, it is evident that effective cancer therapy will in most cases have to be combinatorial. However, in the clinic, combinations of more than three or four drugs has proven to be cumbersome. Additionally, inhibition of all these survival pathways alone or in combination with pro-apoptotic agents might well be associated with severe damage to normal tissues. Finally, it is prohibitively expensive and very time consuming to test *in vivo*

all of the possible combinations of drug doses and schedules when the number of drugs exceeds three. In summary, there are many reasons to screen *in vitro* to determine the minimum set of drugs sufficient to induce apoptosis.

Studies of drug interaction usually reveal that two drugs can be synergistic, additive or antagonistic depending on the concentrations used and the ratio of one drug to another. We will use tissue culture experiments to define the optimal combinations of drugs. These combinations will then be used in Specific AIM II to design dose-finding experiments *in vivo*. The experiments in Specific AIM I are designed not only to identify treatment combinations that work well *in vitro* but equally important to identify and eliminate those that do not work *in vitro*.

Research strategy *(see flowchart).*



1. We will optimize protocols for inhibition of individual survival signaling pathways in LNCaP cells. Pharmacological inhibitors will be used to prevent activation or block the function of Akt, EGFR, androgen and PKA. Expression of Bcl-xL will be decreased using an antisense approach.

2. Next we will determine if inhibition of all of these pathways simultaneously can induce apoptosis in LNCaP cells.

3. If so we will determine what survival pathways **must** be inhibited and what combination of inhibitors is optimal for induction of apoptosis. We will especially look for the possibility that "androgen ablation" can induce apoptosis efficiently, when other survival pathways are inhibited.

4. We will also test whether the addition of pro-apoptotic factors to an optimal combination of survival pathway inhibitors will produce synergistic tumor cell kill. Our expectation is that we can improve the effectiveness of pro-apoptotic therapies by inhibiting survival pathways.

5. Since this proposal is directed toward clinical application of tissue culture results, we will optimize the combination of anti-survival and pro-apoptotic agents to achieve maximal cell kill with minimal number and minimal concentrations of individual components.

1. Optimize inhibition of individual survival pathways.

Since we will be adding drugs to inhibit various survival pathways thought to be important *in vivo*, it is important that these pathways be activated in our tissue culture experiments. To this end, we will use an enriched medium composed of T medium supplemented with the following:

1) 10% fetal calf serum
2) 10 nM EGF
3) 1 μM Forskolin
4) 10 μM arachidonic acid bound to serum albumin

LNCaP cells also respond to androgen and this may contribute to cell survival. We and others typically use the non-metabolizable androgen, R1881. However, the optimum concentration of R1881 in this new enriched medium is not known. For this reason, we will obtain a dose-response curve for R1881 in this new medium, using increase in cell number as the end point. The optimal concentration of R1881 will then be used in subsequent experiments, save those where the impact of androgen withdrawal is being tested.

In the rest of this proposal we will refer to this as "rich" medium.

The justification for each of these additions is outlined in the Background Section.

The goal in these experiments will be to determine how much drug it takes to block the target. This information will enable us to determine whether inhibition of individual pathways is necessary for induction of apoptosis. Those pathways that are unnecessary or ineffective will be dropped from the more combinatorial analysis described in Section I. 2.

The drug, the target and the assay to be used to assess its inhibition are described below.

Inhibiting Akt..

Activity of Akt in LNCaP can be inhibited by wortmannin (100 nM) or LY294002 (25 μM). We will use wortmannin because it is already in preclinical development in the NCI RAID program. Our previous experience shows that the inhibitory effect of wortmannin in other cell types lasts for 6h. We will make dose-response curves and perform time courses to determine the optimal regimen of treatment. Efficiency of inhibition will be determined by Western blots with antibodies recognizing p-Akt and by immune complex kinase reactions. Akt inhibitors are under development at Bristol-Myers and when those become available we will examine them similarly (see letter of collaboration in Appendix).

Inhibiting EGFR and other HER family receptors.

In the context of this proposal, the pan HER family inhibitor, PD180805 will be used because signaling via other members of the HER family (e.g.HER2) may be contributing to progression of prostate cancer. An additional advantage is that PD180805 is already in clinical trials. We will make dose-response curves and perform time courses to find the optimal regimens of inhibition. Tyrosine kinase activities of HER family members will be followed by detecting autophosphorylation of EGFR and HER2 by Western blots with phosphotyrosine specific antibodies. We already know that AG1478, a specific inhibitor of EGFR tyrosine kinase activity, can prevent the survival effects of EGF. Therefore, we will use AG1478 as a positive control for the PD180805 experiments.

Inhibiting survival mediated by PKA.

Inhibition of PKA can be achieved by H89 or 8-Cl-cAMP. Efficacy of PKA inhibition will be monitored by *in vitro* immune complex kinase assays. However, because PKA is involved in so many biological processes its inhibition may produce severe side effects. For example, the PKA inhibitor, 8-Cl-cAMP, caused hypercalcemia in patients. If we find that survival via PKA must be inhibited in subsequent xenograft experiments we will attempt to do it downstream of PKA.

Survival effects of PKA activation may be mediated by phosphorylation of Bad, but we did not observe forskolin-induced Bad phosphorylation in LNCaP cells (unpublished). Another possibility is signaling by PKA via Rap-1 and B-Raf. Indeed Raf activity has been connected with survival signaling in other cell types. Since inhibition at the level of B-Raf is less likely to cause severe side effects, we will determine whether antisense B-Raf will interfere with the survival effect of forskolin. Decrease in B-Raf levels will be monitored by Western blot. If successful this approach will be used for optimization in stage 3 and in subsequent experiments.

Inhibiting expression of Bcl-xL.

We will assess effects of antisense oligos targeting Bcl-xL because this protein is most abundant in LNCaP compared with other anti-apoptotic proteins of this family.

Inhibiting androgen action.

We will monitor PSA expression. We have already seen a 3-fold reduction in PSA promoter activity by 30 μM Casodex.

2. Assess if simultaneous inhibition of survival pathways is sufficient to induce apoptosis in LNCaP cells.

LNCaP cells will be transferred into the "rich" medium. Drugs will be combined, each at doses required to completely block the target. For example, wortmannin, PD180805, H89 and Bcl-xL antisense would be combined. This should result in massive apoptosis (other possible outcomes are discussed below). Assuming this is the case, a dose-response curve using serial dilutions of this combination will be performed. This will allow us to identify the $ED_{90\text{-}100}$ for this combination.

Survival will be determined by MTT assay and apoptosis will be assessed by measuring caspase activity with fluorogenic substrates or nucleosome formation. These assays will be performed in 96-well plate format using 2-color fluorescent reader available in PI laboratory.

Because bone is the most frequent location of prostate carcinoma metastases we will try to recreate similar conditions *in vitro*. We will use mixed cultures of LNCaP cells with bone

stromal MS cells in "rich" media. We will test the best drug combinations from the last stage in these mixed cultures. LNCaP cells expressing GFP will be used to distinguish apoptosis in LNCaP cells from MS cells. LNCaP/GFP cells are available from Leland Chung, a SPORE participant and director of the Small Animal, Imaging and Tissue Culture Core. These cells display the same characteristics *in vitro* and in xenograft models as regular LNCaP. As a primary assay for apoptosis we will measure the number of LNCaP/GFP cells in mixed cultures using a fluorescent microplate reader available in the PI's laboratory. Subsequently apoptosis will be assessed for each cell line independently by counting fragmented nuclei (method routinely used in co-PI laboratory) or by two-color FACS analysis separately measuring subdiploid population in GFP-positive and GFP-negative cells.

3. Determine what survival pathways *must* be inhibited and the optimal concentrations and combinations.

Our stated goal is to find the minimum combination of drugs able to kill all of the cancer cells via apoptosis. The next set of experiments will seek to identify those drugs in the combination that can be eliminated without compromising tumor cell kill. This will be accomplished by eliminating, in rotation, each drug from one combination.

For example we may find that a combination of 1) PI3K inhibitor wortmannin, 2) HER inhibitor PD180805, 3) PKA inhibitor H89, 4) Bcl-xL antisense and 5) Casodex, induces apoptosis. The next experiment will use five combinations of four treatments: 1,2,3,4; 1,2,3,5; 1,2,4,5; 1,3,4,5; 2,3,4,5; then, with three treatments, and so on until the minimal set of drugs needed to maintain tumor cell kill has been identified.

Once the minimum set of drugs has been identified, the next goal will be to define the optimal ratio of the various drugs in the combination. This will be done by examining which drug ratios are associated with maximal impact on the cancer cells. Statistical methods for making this determination are described below.

Analysis of survival and apoptosis will be performed in semi-automated 96-well format.

4. Assess if pro-apoptotic factors together with inhibitors of survival signals will produce synergistic effect.

We will use Taxol and MK886 to facilitate induction of apoptosis in LNCaP cells. We expect a significant degree of synergy between these drugs because they operate via different "arms" of proapoptotic signaling. Taxol is a microtubule polymerizing drug that is commonly used to treat prostate cancer, typically in combination with estramustine. At 10 nM (an ED_{50}) it induces apoptosis in LNCaP cells growing in 10% FCS while higher concentrations can specifically decrease Bcl-xL expression. Most probably Taxol induces apoptosis via the "mitochondrial" arm.

The lipoxygenase inhibitor MK886 induces apoptosis in LNCaP cells by facilitating translocation of FAS and FAS ligand to the plasma membrane and subsequent activation of FAS signaling. This observation was recently made in the PI's laboratory. Signaling via the FAS pathway may play a role in inducing apoptosis in prostate cells after androgen withdrawal (see Background). Expression of FAS and FAS ligand is preserved even at advanced stages of prostate cancer. Also, treatment of LNCaP cells with cycloheximide and TNF-α can induce apoptosis in the presence of survival agonists. Thus, finding a way of activating FAS signaling is a promising avenue in search of treatment for advanced prostate cancer.

MK886 was already tested in animal experiments. If combinations with other drugs show positive results but are not effective in xenograft models we will screen the library of

chemicals for compounds inducing translocation of FAS. FAS translocation will be followed by FACS with anti-FAS antibodies.

We are planning to assess if treatment with pro-apoptotic agents will produce synergy in induction of apoptosis when combined with the "anti-survival cocktail" and if so determine optimal concentrations which will be used for experiments in xenografts

Experimental approaches and methods:

Cell Lines.

LNCaP may be obtained from several sources that may not be identical. During the establishment of our PO1 grant, we settled on the LNCaP cell line maintained by Dr. Leland Chung. For these experiments, we will use LNCaP cells that have been grown in the presence of 0.1 nM R1881 for more than three months.

Apoptosis assays.

We will use apoptosis assays that are adaptable to semi-automatic processing in a 96 well format. We have found that assays for Caspase 3 activation and nucleosome formation correlate well with apoptotic nuclei and TUNEL in LNCaP. Both the caspase 3 and the nucleosome assay can be performed in 96 well plates using a Molecular Dynamics 2-color fluorescent plate reader available in the PI's laboratory. This will permit us to rapidly examine the effectiveness of these various combinations of agents in a semi-automated procedure.

Synergy analysis in vitro

We have previously published an analysis of synergy between Taxol and quinacrine in prostate cancer cells. In that paper, our analysis primarily rested on the median effect principle [48]. In these experiments we will be testing combinations of novel agents that may create complex dose-response curves not amenable to analysis using the median effect principle. For this reason, we will start by examining the entire 3-D dose-response curve. The first step is to determine the dose-response curve for each individual drug. Then the dose-response curve for one drug is repeated at concentrations of the second drug that cover the second drug's entire dose range. The result is a three dimensional plot with the concentrations of the two drugs on the x and y axis and the response on the Z axis. The advantage of this approach is that it shows the result for every possible combination of the two drugs. The one disadvantage is that it requires the performance of a large number of dose-response curves. However, with 96 well plates and using modern techniques of automation this is no longer difficult. At the end of these experiments, we will assess whether analysis by median effect principle is valid. If problems are detected, we will proceed using the parametric response surface approach of Greco, et al [49]. This analysis will be performed using the Gauss program by Aptech Systems, Inc. These analyses will be done in collaboration with Dr. Conaway of the Biostatistics Core.

This approach can also be used to examine the interaction between two different combinations of drugs. In this case, the ratio of the individual drugs in each combination must be fixed and dose-response curves generated for each fixed ratio combination individually. Following this, a complete dose-response surface is generated in the same way as it is with two drugs.

Outcomes and Alternatives.

1. Inhibition of some survival pathways is incomplete.

It is important to make sure that all anti-survival agents are working as expected because if we are not certain about the efficiency of target inhibition, interpretation of negative results in subsequent experiments will be impossible. We are almost certain that we will not encounter problems with inhibitors of PI3K, PKA, Casodex and HER family because they already were tested in tissue culture and *in vivo* in our laboratory or in those of our collaborators.

We have not tested the antisense constructs in our own laboratory, however the oligos we propose to use have all been tested in LNCaP cells.

In the unlikely event we fail to inhibit survival targets pharmacologically or with antisense, there is a wealth of alternatives that can be used, particularly in the HER family and in the PI3 Kinase pathway. For example, Warner-Lambert has developed several inhibitors of the HER family that differ in specificity and mechanism of action and Astra Zeneca has a drug (Iressa/ZD1839) that is now in clinical trials. Lilly has several additional PI3 Kinase inhibitors and Bristol-Myers is developing Akt inhibitors. Antisense oligos represent an additional approach to inhibiting PKA. Since Taxanes induce phosphorylation of Bcl-xL and Bcl-2 we expect that the effects of these drugs will be congruent with the antisense oligos against Bcl-xL and that the protein will be functionally inhibited as well as down-regulated.

2. Inhibition of all targeted survival pathways does not induce apoptosis in LNCaP cells.

Essentially this will mean that there are some survival signals in the environment provided by MS or serum which are not inhibited by our combination of anti-survival drugs.

2.1 First, as we proposed above, we will examine the effect of inhibiting survival signaling on the activity of pro-apoptotic agents. For example at 10 nM, Taxol is at an ED_{50} and alone is sufficient to induce apoptosis in LNCaP in the presence of serum, and our hypothesis is that inhibition of survival signaling will shift the dose-response curve for this drug to the left. We expect that by using a variety of pro-apoptotic agents and inhibitors of survival signaling we will be able to identify a combination with a synergistic effect on cell death.

2.2. If inhibition of the known survival pathways does not potentiate the effects of treatment with pro-apoptotic agents, it implies that other, perhaps novel, survival mechanisms are limiting the effectiveness of the cytotoxic drugs. This opens up an avenue of translational research in which a clinically-directed question leads to fundamental investigations.

It is possible that transcription factors that can control an anti-apoptotic program, such as NFκB or CREB, have induced a survival pathway that we have not inhibited. It is possible to test this by use of dominant negative mutants.

Another approach is to determine which of the components of the "rich" medium is responsible for triggering the protective effect. We will test the consequences on cell survival of removing, in turn, MS cells, EGF, forskolin, or serum. Most probably the new survival factor will come from serum or bone cells. If so, we will determine whether a defined mixture of cytokines can provide a similar survival effect. If we identify one or a few cytokines that can mimic the effects of serum or MS cells, we have the expertise to identify the relevant signaling pathways. If we cannot identify a defined cytokine we would contemplate purification of an unknown survival factor from serum (or media conditioned by MS cells), using the survival assay as readout. However, as this would be a long-term, high-risk procedure, we would first determine whether the same problems occur *in vivo*.

Alternatively we can use LNCaP growing in "rich" medium to screen the natural products library of chemical compounds using the Natural Products Discovery Core. The active compounds will be used for anti-survival experiments and can also be used for purification of targets.

Specific AIM II.

Examine the most promising combinations of agents in xenograft models. This will involve examining not only effects on tumor size and PSA, but also thorough analysis of the amount and distribution of apoptotic bodies in the tumor.

Rationale

The overall goal of this proposal is to select the minimal set of drugs that can effectively cause apoptosis, resulting in an increase in the complete remission rate. In AIM I, we made the assumption that any drug or drug combination that was inactive in tissue culture is unlikely to be active *in vivo*. In contrast, any drug or drug combination active in tissue culture might well fail *in vivo* for any number of reasons. Perhaps of greatest concern, the *in vivo* environment immerses the cancer cells in a rich mixture of cytokines and adhesion molecules that can not yet be duplicated in tissue culture.

While human tumor xenograft models still fall short of testing a human tumor in a human host, they are nevertheless a step up from tissue culture assays in clinical relevance. However, even in a xenograft, regulatory signals that are characteristic of a tumor in its normal environment may not be present. To minimize this problem we propose to use four implantation protocols: (1) with matrigel in the flank; (2) orthotopically; (3) with MS osteoblastic cells in the flank and (4) in the femoral marrow cavity. The first site represents the standard procedure and the simplest system to manipulate and measure. The second site is representative of localized disease. The third and fourth sites provide a measure of effects of the bone environment, because hormone-resistant prostate cancer typically appears in the bone.

In AIM I we identified a minimal set of drugs able to efficiently induce apoptosis of LNCaP. We then used techniques of synergy analysis to identify the optimal ratios of the active agents. In AIM II, we will determine if the combination or combinations identified in AIM I have sufficient efficacy to warrant mounting a clinical trial.

Strategies

We will test the active combination or combinations from AIM I against LNCaP cells growing in immunocompromised mice. The studies planned are those classic to preclinical drug development and include in sequence:

- A dose-ranging study to identify the maximal tolerated dose (MTD) of the combination or combinations.
- The equivalent of a human Phase II study where groups of tumor-bearing mice are treated with the combination or combinations at the MTD. At this point we would assess the ways that different sites of implantation and/or tumor models affect efficacy.
- A dose-ranging study that defines the difference between the dose optimal for antitumor effects compared and the MTD.
- A toxicology study to define the pattern of normal organ injury at the MTD.

It is important in these studies to measure not only tumor response by changes in tumor size and PSA but also by induction of apoptosis, since this is the rationale underlying all the proposed experiments. As described below, apoptosis will be determined both by pathologic examination of biopsies and necropsies and also by a non-invasive imaging technique [50, 51] that we expect to be transportable into human clinical trials.

Animal models are routinely used in preclinical studies in an attempt to anticipate the normal tissues likely to be damaged by a drug or combination of drugs. However, no single

species can be depended upon to reliably predict human toxicity. For example, mice are not very effective at anticipating nausea or neurotoxicity. We will examine the pattern of organ toxicity at the mouse LD_{10} and determine if methods exist to lessen the toxicity of the treatment. If such options exist, we will test them in these animal models to determine if methods that reduce toxicity also compromise therapeutic efficacy. The PI has extensive experience in such matters. For example, he has published extensively on the mechanism of doxorubicin cardiac toxicity and its amelioration.

At the end of our experiments, additional work may be needed to characterize the drug combinations prior to initiating human trials. For example, toxicology in the dog is generally regarded as complementary to mouse toxicology.

Experimental approaches and methods

The initial experiments will determine responses as a result of 14 days of treatment. This time frame was chosen because should be long enough for promising combinations to cause a significant response. A lesser, but still important, goal would be to extend the time until PSA starts to rise exponentially and tumor growth resumes as judged by a rebound in PSA and increasing tumor size.

Dose-finding Experiments

The first mouse experiments with any combination will be a dose-finding study in nontumor-bearing mice to determine the LD_{10} for treatment of 14 days duration. Drugs in the combination will be used at the ratios that proved to offer the greatest synergy *in vitro*. In these dose-finding studies, three mice will be treated at each dose level and the doses in the combination will be increased using the modified Fibonacci sequence commonly used in human Phase I clinical trials until at least one mouse out of three dies. A second set of studies will be done to refine the LD_{10} in mice. Using 10 mice per group, three groups will be treated using the maximal dose from the first set of experiments bracketed by doses + and – 20%.

Antitumor Efficacy Experiments

Using the MTD from the mouse Phase I studies, we will examine the antitumor effectiveness of the combination or combinations that emerged from the experiments in AIM I. The end points of these studies will be maximal decrease in tumor volume and regrowth delay following treatment, with a special emphasis on the proportion of complete remissions. Any mice alive and free of measurable tumor and with an undetectable PSA at 60 days will be regarded as cured.

It has been reported that LNCaP grows best in SCID mice and our experience confirms this observation. For each cell line, 2 $x10^6$ cells, together with Matrigel™, are injected (50% cell suspension in RPMI-1640: 50% Matrigel). Matrigel is used to improve the "take" rate. Tumors are monitored for growth and the mice will be randomized between treatment and control when the tumors are 0.5-1 cm3 or a PSA between 5 and 10 ng/ml.

We plan ten mice per group in the initial experiments. The results of those experiments will be used to design a second round of mouse studies with the number of mice per group governed by power calculations done by our Biostat. Core. Even if no complete responses are seen in the initial round of treatment, we will analyze delayed recurrence and induction of apoptosis (see below) and thus we expect that these experiments will still be informative.

Tumor volume, TV, is calculated according to the formula, TV = (length x width2)/2. This formula has previously been shown to correlate very well with excised tumor weight.

Role of Androgen Withdrawal

LNCaP cells are best termed androgen-responsive, rather than androgen-dependent. In the xenograft model, castration causes a minor growth delay and a transient decline in the serum PSA and growth resumes within 2-3 weeks. While not effective as sole treatment, it is possible and even likely that androgen withdrawal will enhance the activity of drug combinations designed to block survival pathways and provide proapoptotic stimuli. For this reason, we will test the most active combination in intact mice as well as those that undergo castration simultaneous with the initiation of anti-survival therapy.

Orthotopic implantation

Orthotopic implantation is performed routinely in the PI's laboratory. It potentially provides a model more reflective of localized human disease than flank implantation, and orthotopically implanted tumors often differ in response to chemotherapy than tumors implanted in the flank. In second generation experiments, it will be possible to examine metastatic variants of LNCaP implanted orthotopically to determine whether antisurvival treatments block the ability of tumors to spread to defined sites.

Coinnoculation of LNCaP cells and the MS osteoblastic cell line

In AIM I, we will have tested the various promising drug combinations against mixed cultures of LNCaP and the MS osteoblastic cell line. The rationale is that in these mixed cultures, LNCaP may be exposed to a range of survival factors likely to more closely approximate the environment prostate cancer cells encounter when they spread to the bone marrow. We will now extend these experiments to an *in vivo* model. Dr. Leland Chung has characterized a model in which LNCaP cells are co-inoculated with MS cells into the flank of mice and observed enhanced tumor take and growth [45, 46]. He has now developed a system in which GFP-labeled LNCaP cells can be implanted with MS cells. When the mice are sacrificed, the tumor can be removed and the number of GFP-labeled cancer cells detected on frozen section. Our Pathology Core has experience in the use of this technique.

The combination or combinations that show significant activity in the earlier animal models will now be tested with GFP-LNCaP/MS implanted into the flank of mice. Again, the tumors will be allowed to grow to 0.5-1.0 cm^3 and the animals then will be treated for 14 days. End points of these initial experiments will be tumor size and serum PSA. If the tumor shrinkage occurs, we will perform a second experiment and terminate sets of mice over the time interval during which tumor shrinkage occurs. We will use GFP fluorescence to assess the proportion of LNCaP cells in the masses during tumor shrinkage.

Bone marrow implantation

In patients, hormone-resistant or chemotherapy resistant prostate cancer most commonly emerges at sites of bony involvement. Through the Small Animal, Imaging and Tissue Culture Core, we have access to mice in which LNCaP has been implanted into the femoral marrow cavity. Once we have identified and fully characterized a successful drug combination using the models described above, we will test this combination against LNCaP implanted in the femoral marrow. At this site of tumor implantation LNCaP survives only until the marrow is filled, at which point the tumor dies -- approximately 4 weeks. This may be too short a time for us reliably to assess therapeutic response by measurements of tumor growth and PSA. However, we should still be able to determine whether our treatments will induce apoptosis in the bone

environment, either by pathologic examination or Annexin V imaging. In addition, Leland Chung has developed LNCaP derivative cell lines (C4-2, C4-2B) that survive and spread following bone implantation, and these serve as a suitable model for advanced metastatic disease.

Animal Pathology

After dissecting tumor from the mouse, formalin-fixed and paraffin-embedded tissue blocks will be prepared using routine histopathology procedures. One hematoxylin and eosin (H&E) stained slide, and 5 serial 4 μm unstained sections will be prepared from each tissue block. Histopathologic features of the treated tumor will be examined using light microscopy.

The efficacy of the drugs in our combinations will in every case be evaluated so that we can interpret negative results and also so that we can have confidence that positive responses are occurring via the predicted mechanism. The identification of apoptotic cells will be performed using a modified TUNEL method. Because many of the survival signaling events are associated with increased phosphorylation of specific proteins and because phosphorylation-state specific antibodies are now available for a number of these proteins (e.g. EGFR, HER2, Akt-1, MAP Kinase, JNK, Bad) we propose to examine the effectiveness of the drugs in disrupting these phosphorylations, either by performing western blots or immunohistochemistry on the excised tumors. For example, if we find that wortmannin treatment has no effect on tumor survival or induction of apoptosis, we would determine whether this drug inhibited Akt phosphorylation in the xenograft as it did in the cell cultures[18]. Note that we have experience demonstrating the phosphorylation of MAP Kinase in paraffin thin sections of prostate cancers [52] and we expect that similar measurements in xenografts will present a less challenging medium for these measurements.

Before and after the treatment of mouse with the drug, peripheral blood counts, liver function tests (bilirubin, LDH, alkaline phosphatase, hepatic transaminases), and renal function tests (BUN and creatinine) will be performed for each mouse, respectively.

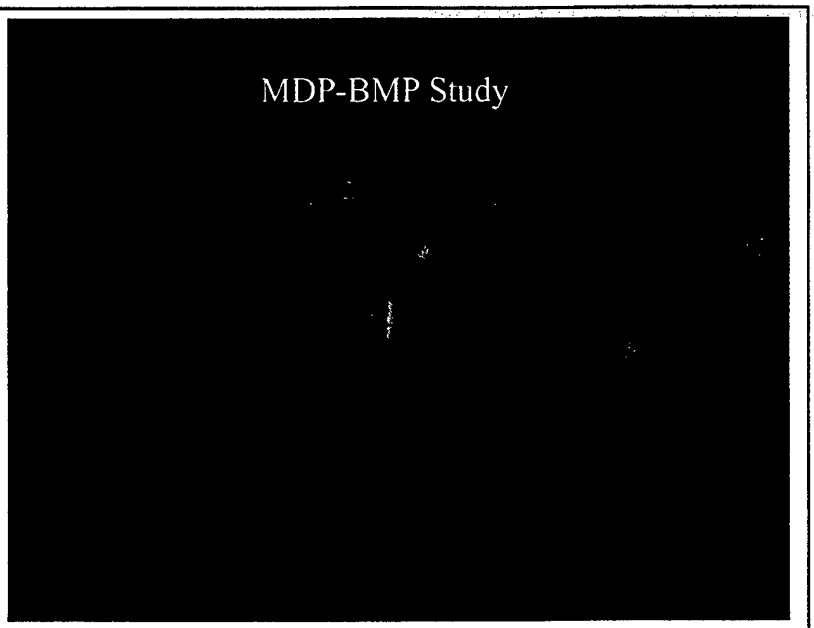
Annexin V Imaging

Because these experiments are designed to aid the conduct of a clinical trial, we have sought a noninvasive indicator of apoptosis that could aid both the xenograft studies and be a useful surrogate marker in clinical trials. Recently, ^{99m}Tc-annexin V has been used to image apoptosis *in vivo* in animal models and we propose to examine the relationship between pathologic assessment of apoptosis and the results of ^{99m}Tc-annexin V imaging. The potential payoff would be a noninvasive imaging technique able to visualize apoptosis in the tumors of patients undergoing treatment with drug combinations designed to enhance apoptosis.

Imaging techniques can be used to noninvasively identify cells undergoing apoptosis. Blankenberg et. al. [50] have shown that the endogenous human protein, annexin V, has a high affinity for phosphatidylserine, a membrane component usually found only on the inside of the cell membrane and which is externalized in cells undergoing apoptosis. Using annexin V that had been radiolabeled with ^{99m}Tc, they found a two- to six-fold increased uptake at sites of apoptotic cell death in three different animal models [50]. The same group has recently demonstrated increased uptake of both radiolabeled and fluorescein labeled annexin V in apoptotic and necrotic cells following intravenous injection of anti-Fas antibody [51].

^{99m}Tc is a radioisotope that can be produced in a bench top radionuclide generator, and emits a 140 keV gamma ray. This, along with its 6-hour half-life, makes it ideally suited for imaging studies. We have availa[illegible] through our Small Animal Imaging and Tissue Culture

We will use this instrumentation with ^{99m}Tc-annexin V to non-invasively image the induction of apoptosis in the xenograft and in normal tissues in response to treatment. The methodology allows us not only to gain a first approximation of the extent of damage to normal tissues, but also to monitor repeated and sequential treatments in real time without sacrificing the animals.



The figure is an example of dual modality images of a rat, injected with a ^{99m}Tc-labeled compound (MDP). The images were obtained 12, 18, and 24 days following the injection of a bone morphogenetic protein (BMP) into the thigh muscle. ^{99m}Tc- MDP is taken up by actively growing bone cells.

Biostatistics

Repeated measures models will be used to explore the effect of combinations of agents on tumor growth and PSA. We have found in other studies of tumor growth and PSA, that modeling ln (tumor size) and ln (PSA) with an AR-1error structure provides an excellent fit to the data. Independent variables in these models will include time effects and main effect and interactions for the factors defining the combination of agents. Random coefficient models could also be used, but we have found that these tend to fit less well. In the random coefficient models, the correlation between observations within an animal are derived from the amount of variation in tumor growth profiles across animals in the population. In experiments that use genetically similar mice, this variation tends to be small and the models tend to underestimate the amount of correlation between observations over time.

Tests of the effects of combinations of agents will be carried out using F-tests. In the repeated measures setting, these tests can be liberal with respect to type I error. In addition, the typical standard errors can underestimate variation. We will use resampling methods such as permutation tests and bootstrap methods to achieve the nominal levels for tests and confidence intervals.

Once the time course of combinations has been studied, The effect of combinations on the amount and distribution of apoptotic bodies in the tumor will be tested using ANOVA methods. Depending on the results of the apoptosis experiments, these experiments may or may not have complete factorial structure. For example, certain combinations of pathway inactivation and pro-apoptosis factors may be demonstrated to be ineffective for apoptosis and will not necessarily be used in the xenograft experiments. We will estimate the effects of the entire combination by computing the average response number and distribution of apoptotic agents) for each combination. The effect of individual components (signal pathway inactivation, pro-apoptotic agent) in the combination will be estimated with contrasts.

necessarily be used in the xenograft experiments. We will estimate the effects of the entire combination by computing the average response number and distribution of apoptotic agents) for each combination. The effect of individual components (signal pathway inactivation, pro-apoptotic agent) in the combination will be estimated with contrasts.

Outcomes and Alternatives:

Our expectation is that inhibiting PI3 Kinase or Akt will have a substantial pro-apoptotic effect when combined with a cytotoxic agent such as Taxol. The fact that there is a powerful selection for loss of PTEN in tumor development provides strong evidence that this pathway plays an important role in survival signaling. We likewise expect that inhibiting the EGF Receptor family will generate even more apoptosis, as these receptors are commonly overexpressed and yet the tumor cells do not necessarily display a higher growth fraction. We suspect that overexpression of EGFR and TGF-α play a role in survival signaling in tumor development.

If we fail to observe enhanced apoptosis even though the drug targets are inhibited, we will excise the tumors that re-grow after treatment, re-culture them and determine in cell culture and in xenografts whether the survivors are enriched for a genetic variant resistant to the induction of apoptosis. One possibility is increased expression of a critical target, such as Bcl-2 or Bcl-xL, and we would evaluate the expression and activity of the known survival pathways that we described in Background. Such a variant could provide a useful tool in second generation experiments (designed as outlined in Aim I).

In addition, such a variant could become a tool for discovery of new survival signaling pathways, for example by procedures analogous to those outlined in project 6: a cDNA library would be made from the resistant cells, transfected into sensitive cells and genes that confer resistance to apoptosis would be identified. We expect this to be beyond the scope of the current proposal, although it would be translational research.

If the tumor that recurs is not stably resistant to pro-apoptotic stimuli, then this implies that the resistance to treatment is a consequence of physiologic heterogeneity and might be overcome by changes in duration, schedule and intensity of treatment. For example, if the resistance is due to cell-cycle stage, then changes in dosing schedule could enhance the percent kill.

Interactions

This Project anticipates strong interactions with all the other therapeutically-oriented Projects: 3, 4, 5 and 6. With Drs. Chung, Zhau and Bostwick (Project 3) we expect that inhibition of survival signaling will enhance the effectiveness of gene therapies, especially those aimed at destroying stroma. Conversely, the technology developed by Dr. Chung opens the door to using adenovirus vectors to deliver genes that interfere with survival signaling. With Drs. Lynch, Macdonald and Theodorescu (Project 4) we will be able to help them assess the role of LPA as a "survival factor" for prostate cancer cells and the efficacy of LPA antagonists as components of a combination chemotherapy protocol. With Drs. Theodorescu, S. Parsons and Brautigan (Project 5) we can examine the ways angiogenesis alters the sensitivity of prostate cancer cells to therapy and whether a VEGF inhibitor promotes apoptosis in cells stressed with therapeutic agents. With Drs. Horwitz, J.T. Parsons and Hecht (Project 6) we anticipate a very close working relationship as genes and compounds that alter cell motility and morphology are identified, since it is well established that integrin signaling is a major survival mechanism for most cells.

Core Usage

We will be very heavy users of the Small Animal, Imaging and Tissue Culture Core. With the help of this core we will identify cell culture and xenograft models, assess their appropriateness and utilize them experimentally. In addition this Core will work with us in applying the methodology to detect apoptosis in xenografts by non-invasive imaging techniques. We also will be heavily dependent on the Biostatistics Core for analysis of the multiple variables involved with our experimental approaches and for the detection of synergisms. Although we do not at this point plan to use the Natural Products Discovery Core to screen for new pro-apoptotic agents we will be deeply involved in all the projects (mentioned above) that use this Core to identify new inhibitors of signaling pathways. In addition, it is not improbable that we will also wish to utilize the Core for identifying new inhibitors of survival signaling. Such inhibitors can be important tools in dissecting out previously unrecognized survival signaling pathways as well as providing a proof of principle for further drug discovery. Finally, the Pathology Core will be essential in analysis of xenograft responses to our pro-apoptotic therapies. In addition this Core has the potential for providing patient samples that could provide us with clinical correlations relevant to our research, such as frequency of apoptosis in response to androgen withdrawal and/or radiation and/or Taxol.

Human subjects. None.

Vertebrate animals. Tumor studies require the use of rodent hosts. There are no viable alternatives. All animal studies occur under approved protocols.

UNIVERSITY OF
VIRGINIA

August 26, 1999

Dr. Charles Myers
Department of Internal Medicine
Box 334

Dear Dr. Myers:

Re: Protocol No. 2540

Initial Review Date: 08-27-97
First Annual Review: 09-15-98
Second Annual Review: 08-17-99

The Animal Care and Use Committee (ACUC) is pleased to inform you that full approval has been given for:

_____ ***New Protocol*** [*approved for a three year term with annual reviews; the month in which the approval was given becomes the anniversary month for Annual Review and Continuation after Three Years*]

_____ ***Modification*** [*approved and added to protocol*]

__✓__ ***Annual Review*** [*approved each year in anniversary month during three year term*]

_____ ***Continuation after Three Years*** [*approved for additional three year term with annual reviews*]

A copy of the approved protocol is enclosed for your reference. A notification from the ACUC office to the Primary Investigator and to the Contact Person will be sent thirty to sixty days prior to the anniversary month. It is the responsibility of the Primary Investigator to ensure that if the protocol(s) is (are) to continue without interruption, Annual Review and Continuation after Three Year proposals must be approved before the end of the anniversary month.

Sincerely,

Sanford H. Feldman, Ph.D., D.V.M.
Vice Chairman
Animal Care and Use Committee

Enclosure: Contact Person
(Please post a copy of the approved protocol in your laboratory for technical reference.)

Literature Cited:

1. Crawford, E.D., M.A. Eisenberger, D.G. McLeod, J.T. Spaulding, R. Benson, F.A. Dorr, B.A. Blumenstein, M.A. Davis, and P.J. Goodman, *A controlled trial of leuprolide with and without flutamide in prostatic carcinoma [published erratum appears in N Engl J Med 1989 Nov 16;321(20):1420].* N Engl J Med, 1989. 321(7): p. 419-24.

2. Westin, P., P. Stattin, J.E. Damber, and A. Bergh, *Castration therapy rapidly induces apoptosis in a minority and decreases cell proliferation in a majority of human prostatic tumors.* Am J Pathol, 1995. 146(6): p. 1368-75.

3. Gleave, M.E., S.L. Goldenberg, E.C. Jones, N. Bruchovsky, and L.D. Sullivan, *Biochemical and pathological effects of 8 months of neoadjuvant androgen withdrawal therapy before radical prostatectomy in patients with clinically confined prostate cancer [see comments].* J Urol, 1996. 155(1): p. 213-9.

4. Furuya, Y., S. Krajewski, J.I. Epstein, J.C. Reed, and J.T. Isaacs, *Expression of Bcl-2 and the Progression of Human and Rodent Prostatic Cancers.* Clinical Cancer Research, 1996. 2(2): p. 389-398.

5. Krajewska, M., S. Krajewski, J.I. Epstein, A. Shabaik, J. Sauvageot, K. Song, S. Kitada, and J.C. Reed, *Immunohistochemical Analysis of Bcl-2, Bax, Bcl-X, and Mcl-1 Expression in Prostate Cancers.* American Journal of Pathology, 1996. 148(5): p. 1567-1576.

6. Stattin, P., P. Westin, J.E. Damber, and A. Bergh, *Short-Term Cellular Effects Induced By Castration Therapy in Relation to Clinical Outcome in Prostate Cancer.* British Journal of Cancer, 1998. 77(4): p. 670-675.

7. Paterson, R.F., M.E. Gleave, E.C. Jones, J.T. Zubovits, S.L. Goldenberg, and L.D. Sullivan, *Immunohistochemical analysis of radical prostatectomy specimens after 8 months of neoadjuvant hormonal therapy.* Molecular Urology., 1999. 3(3): p. 277-285.

8. Kajiwara, T., T. Takeuchi, T. Ueki, N. Moriyama, K. Ueki, T. Kakizoe, and K. Kawabe, *Effect of bcl-2 overexpression in human prostate cancer cells in vitro and in vivo.* International Journal of Urology., 1999. 6(10): p. 520-525.

9. Miayake, H., A. Tolcher, and M.E. Gleave, *Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense Bcl-2 oligodeoxynucleotides.* Journal of the National Cancer Institute., 2000. 92(1): p. 34-41.

10. Dorai, T., H. Perlman, K. Walsh, A. Shabsigh, E.T. Goluboff, C.A. Olsson, and R. Buttyan, *A recombinant defective adenoviral agent expressing anti-bcl-2 ribozyme promotes apoptosis of bcl-2-expressing human prostate cancer cells.* International Journal of Cancer., 1999. 82(6): p. 846-852.

11. Haldar, S., J. Chintapalli, and C.M. Croce, *Taxol Induces Bcl-2 Phosphorylation and Death of Prostate Cancer Cells.* Cancer Research, 1996. 56(6): p. 1253-1255.

12. Liu, Q.Y. and C.A. Stein, *Taxol and Estramustine-Induced Modulation of Human Prostate Cancer Cell Apoptosis Via Alteration in Bcl-X(L) and Bak Expression.* Clinical Cancer Research, 1997. 3(11): p. 2039-2046.

13. Lebedeva, I.V. and C.A. Stein, *Bcl-xL-directed oligonucleotides in prostate and bladder carcinoma cell lines.* Antisense & Nucleic Acid Drug Development., 1999. 9(4): p. 381-382.

14. Khwaja, A., *Akt is more than just a Bad kinase [news; comment].* Nature, 1999. 401(6748): p. 33-4.

15. McMenamin, M.E., P. Soung, S. Perera, I. Kaplan, M. Loda, and W.R. Sellers, *Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage.* Cancer Res, 1999. 59(17): p. 4291-6.

16. Davies, M.A., D. Koul, H. Dhesi, R. Berman, T.J. McDonnell, D. McConkey, W.K. Yung, and P.A. Steck, *Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN.* Cancer Res, 1999. 59(11): p. 2551-6.

17. Gunther, R., H.K. Abbas, and C.J. Mirocha, *Acute pathological effects on rats of orally administered wortmannin- containing preparations and purified wortmannin from Fusarium oxysporum.* Food Chem Toxicol, 1989. 27(3): p. 173-9.

18. Carson, J.P., G. Kulik, and M.J. Weber, *Antiapoptotic signaling in LNCaP prostate cancer cells: A survival signaling pathway independent of phosphatidylinositol 3 '-kinase and Akt/protein kinase B.* Cancer Research, 1999. 59(7): p. 1449-1453.
19. Scher, H.I., A. Sarkis, V. Reuter, D. Cohen, G. Netto, D. Petrylak, P. Lianes, Z. Fuks, J. Mendelsohn, and C. Cordoncardo, *Changing Pattern of Expression of the Epidermal Growth Factor Receptor and Transforming Growth Factor Alpha in the Progression of Prostatic Neoplasms.* Clinical Cancer Research, 1995. 1(5): p. 545-550.
20. Adam, R.M., J.G. Borer, B.J. Williams, J.A. Eastham, K.R. Loughlin, and M.R. Freeman, *Amphiregulin is coordinately expressed with heparin-binding epidermal growth factor-like growth factor in the interstitial smooth muscle of the human prostate.* Endocrinology., 1999. 140(12): p. 5866-5875.
21. Seth, D., K. Shaw, J. Jazayeri, and P.J. Leedman, *Complex post transcriptional regulation of EGF-receptor expression by EGF and TGF-alpha in human prostate cancer cells.* British Journal of Cancer., 1999. 80(5-6): p. 657-669.
22. Arai, Y., T. Yoshiki, and O. Yoshida, *C-Erbb-2 Oncoprotein - a Potential Biomarker of Advanced Prostate Cancer.* Prostate, 1997. 30(3): p. 195-201.
23. Morote, J., I. de Torres, C. Caceres, C. Vallejo, S. Schwartz, and J. Reventos, *Prognostic value of immunohistochemical expression of the c-erbB-2 oncoprotein in metastasic prostate cancer.* International Journal of Cancer., 1999. 84(4): p. 421-425.
24. Qiu, Y., L. Ravi, and H.J. Kung, *Requirement of Erbb2 For Signalling By Interleukin-6 in Prostate Carcinoma Cells.* Nature, 1998. 393(6680): p. 83-85.
25. Chung, T.D., J.J. Yu, M.T. Spiotto, M. Bartkowski, and J.W. Simons, *Characterization of the role of IL-6 in the progression of prostate cancer.* Prostate, 1999. 38(3): p. 199-207.
26. Prewett, M., P. Rockwell, R.F. Rockwell, N.A. Giorgio, J. Mendelsohn, H.I. Scher, and N.I. Goldstein, *The biologic effects of C225, a chimeric monoclonal antibody to the EGFR, on human prostate carcinoma.* J Immunother Emphasis Tumor Immunol, 1996. 19(6): p. 419-27.
27. Ye, D., J. Mendelsohn, and Z. Fan, *Androgen and epidermal growth factor down-regulate cyclin-dependent kinase inhibitor p27Kip1 and costimulate proliferation of MDA PCa 2a and MDA PCa 2b prostate cancer cells.* Clin Cancer Res, 1999. 5(8): p. 2171-7.
28. Burchardt, T., M. Burchardt, M.W. Chen, Y.C. Cao, A. De la Taille, A. Shabsigh, O. Hayek, T. Dorai, and R. Buttyan, *Transdifferentiation of prostate cancer cells to a neuroendocrine cell phenotype in vitro and in vivo.* Journal of Urology., 1999. 162(5): p. 1800-1805.
29. Jongsma, J., M.H. Oomen, M.A. Noordzij, W.M. Van Weerden, G.J.M. Martens, T.H. van der Kwast, F.H. Schroder, and G.J. van Steenbrugge, *Kinetics of neuroendocrine differentiation in an androgen-dependent human prostate xenograft model.* American Journal of Pathology, 1999. 154(2): p. 543-551.
30. Jongsma, J., M.H.A. Oomen, M.A. Noordzij, J.C. Romijn, T.H. van der Kwast, F.H. Schroder, and G.J. van Steenbrugge, *Androgen-independent growth is induced by neuropeptides in human prostate cancer cell lines.* Prostate., 2000. 42(1): p. 34-44.
31. Wu, J.T., T.L. Wu, C.P.Y. Chang, K.C. Tsao, and C.F. Sun, *Different patterns of serum chromogranin A in patients with prostate cancer with and without undergoing hormonal therapy.* Journal of Clinical Laboratory Analysis., 1999. 13(6): p. 308-311.
32. Chen, T.S., R.W. Cho, P.J.S. Stork, and M.J. Weber, *Elevation of cyclic adenosine 3 ',5 '-monophosphate potentiates activation of mitogen-activated protein kinase by growth factors in LNCaP prostate cancer cells.* Cancer Research, 1999. 59(1): p. 213-218.
33. Vossler, M.R., H. Yao, R.D. York, M.G. Pan, C.S. Rim, and P.J. Stork, *cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway.* Cell, 1997. 89(1): p. 73-82.
34. Harada, H., B. Becknell, M. Wilm, M. Mann, L.J. Huang, S.S. Taylor, J.D. Scott, and S.J. Korsmeyer, *Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A.* Mol Cell, 1999. 3(4): p. 413-22.
35. Chijiwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka, *Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly*

synthesized selective inhibitor of cyclic AMP-dependent protein kinase. N-[2-(p-bromocinnamylamino)ethyl]-5- isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem, 1990. 265(9): p. 5267-72.

36. Rokhlin, O.W., G.A. Bishop, B.S. Hostager, T.J. Waldschmidt, S.P. Sidorenko, N. Pavloff, M.C. Kiefer, S.R. Umansky, R.A. Glover, and M.B. Cohen, *Fas-Mediated Apoptosis in Human Prostatic Carcinoma Cell Lines.* Cancer Research, 1997. 57(9): p. 1758-1768.

37. Liu, W.Y., M.A. Rubin, C. Omene, S. Lederman, and C.A. Stein, *Fas Ligand Is Constitutively Secreted By Prostate Cancer Cells in Vitro.* Clinical Cancer Research, 1998. 4(7): p. 1803-1811.

38. Woolveridge, I., M.F. Taylor, F.C. Wu, and I.D. Morris, *Apoptosis and related genes in the rat ventral prostate following androgen ablation in response to ethane dimethanesulfonate.* Prostate, 1998. 36(1): p. 23-30.

39. Suzuki, A., A. Matsuzawa, and T. Iguchi, *Down regulation of Bcl-2 is the first step on Fas-mediated apoptosis of male reproductive tract.* Oncogene, 1996. 13(1): p. 31-7.

40. de la Taille, A., M.W. Chen, A. Shabsigh, E. Bagiella, A. Kiss, and R. Buttyan, *Fas antigen/CD-95 upregulation and activation during castration-induced regression of the rat ventral prostate gland.* Prostate., 1999. 40(2): p. 89-96.

41. Ghosh, J. and C.E. Myers, *Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells.* Proc Natl Acad Sci U S A, 1998. 95(22): p. 13182-7.

42. Albanese, J. and N. Dainiak, *Ionizing radiation alters Fas antigen ligand at the cell surface and on exfoliated plasma membrane-derived vesicles: Implications for apoptosis and intercellular signaling.* Radiation Research., 2000. 153(1): p. 49-61.

43. Uslu, R., N. Borsellino, P. Frost, H. Garban, C.P. Ng, Y. Mizutani, A. Belldegrun, and B. Bonavida, *Chemosensitization of human prostate carcinoma cell lines to anti-fas- mediated cytotoxicity and apoptosis.* Clin Cancer Res, 1997. 3(6): p. 963-72.

44. Dawson, N.A., W.D. Figg, M.R. Cooper, O. Sartor, R.C. Bergan, A.M. Senderowicz, S.M. Steinberg, A. Tompkins, B. Weinberger, E.A. Sausville, E. Reed, and C.E. Myers, *Phase II trial of suramin, leuprolide, and flutamide in previously untreated metastatic prostate cancer.* J Clin Oncol, 1997. 15(4): p. 1470-7.

45. Chung, L.W., *The role of stromal-epithelial interaction in normal and malignant growth.* Cancer Surv, 1995. 23: p. 33-42.

46. Chung, L.W., M.E. Gleave, J.T. Hsieh, S.J. Hong, and H.E. Zhau, *Reciprocal mesenchymal-epithelial interaction affecting prostate tumour growth and hormonal responsiveness.* Cancer Surv, 1991. 11: p. 91-121.

47. Saunders, M.P., A.J. Salisbury, K.J. O'Byrne, L. Long, R.M. Whitehouse, D.C. Talbot, E.B. Mawer, and A.L. Harris, *A novel cyclic adenosine monophosphate analog induces hypercalcemia via production of 1,25-dihydroxyvitamin D in patients with solid tumors.* J Clin Endocrinol Metab, 1997. 82(12): p. 4044-8.

48. de Souza, P.L., M. Castillo, and C.E. Myers, *Enhancement of paclitaxel activity against hormone-refractory prostate cancer cells in vitro and in vivo by quinacrine.* Br J Cancer, 1997. 75(11): p. 1593-600.

49. Greco, W.R., G. Bravo, and J.C. Parsons, *The search for synergy: a critical review from a response surface perspective.* Pharmacol Rev, 1995. 47(2): p. 331-85.

50. Blankenberg, F.G., P.D. Katsikis, J.F. Tait, R.E. Davis, L. Naumovski, K. Ohtsuki, S. Kopiwoda, M.J. Abrams, M. Darkes, R.C. Robbins, H.T. Maecker, and H.W. Strauss, *In vivo detection and imaging of phosphatidylserine expression during programmed cell death.* Proc Natl Acad Sci U S A, 1998. 95(11): p. 6349-54.

51. Blankenberg, F.G., P.D. Katsikis, J.F. Tait, R.E. Davis, L. Naumovski, K. Ohtsuki, S. Kopiwoda, M.J. Abrams, and H.W. Strauss, *Imaging of apoptosis (programmed cell death) with 99mTc annexin V.* J Nucl Med, 1999. 40(1): p. 184-91.

52. Gioeli, D., J.W. Mandell, G.R. Petroni, H.F. Frierson, Jr., and M.J. Weber, *Activation of mitogen-activated protein kinase associated with prostate cancer progression.* Cancer Res, 1999. 59(2): p. 279-84.