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13. ABSTRACT *(Maximum 200 Words)*

Fatty acid synthase (FAS) performs the anabolic conversion of dietary carbohydrate or protein to fat. FAS expression is low in most normal tissues, but is elevated in many human cancers, including androgen sensitive and androgen independent prostate cancer. This project seeks to characterize androgen mediated and androgen independent mechanisms for fatty acid synthesis pathway activation during prostate cancer progression, using cell culture and animal models of prostate cancer, and analyzing human tumor tissue in parallel. It further seeks to evaluate the potential therapeutic utility of FAS inhibitors for prostate cancer in preclinical models. Progress in the first year includes development and validation of cell culture and xenograft model systems for FAS expression and activity, and evaluation of FAS inhibitor efficacy *in vitro* and in three tumor xenograft models. Metabolic labeling studies of human prostate carcinoma tissues have confirmed functional activation of the fatty acid synthetic pathway in clinical disease. The data suggest that FA synthesis provides an important functional aspect of the malignant phenotype in prostate cancer, perhaps supporting cell growth or survival. FAS expression is upregulated by alternate signaling pathways important for prostate cancer growth under androgen withdrawal, suggesting that FAS may serve as a novel target for anti-metabolite therapy in prostate cancer.

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INTRODUCTION: Prostate cancer represents the second leading cause of death from cancer in American men. Elevated expression of the biosynthetic enzyme, fatty acid synthase (FAS), occurs in most biologically aggressive prostate cancers, and corresponds to increased fatty acid (FA) synthetic activity. While FAS expression is androgen responsive, it persists or is reactivated in tumors after androgen ablation. This malignancy-associated cellular function represents a novel therapeutic target. The dependence of prostate cancer on FAS activity is demonstrated in both cell culture and xenograft tumor models: selective inhibition of FAS by anti-metabolite drugs significantly reduces cell growth and survival. These observations lead to the hypothesis that FAS expression/FA synthesis is functionally critical in prostate carcinogenesis and malignant progression, probably through support of cell growth and survival. FAS expression may be upregulated by multiple alternate signaling pathways important for prostate cancer growth. The activity observed in model systems predicts that FA synthesis inhibition will be cytotoxic to all prostate carcinomas that have activated the pathway.

Specific Aim 1: To determine 1] the FAS expression/activity of prostate carcinoma model systems under androgen stimulated, acutely androgen deprived, chronically androgen deprived and androgen independent conditions, 2] the relative contributions of androgen mediated versus androgen independent mechanisms for FA pathway activation under androgen deprived conditions, and the functional significance of insulin-like growth factor stimulation of fatty acid synthase.
Specific Aim 2: To 1] characterize the susceptibility of prostate cancer models to FAS inhibition and 2] optimize the use of FAS inhibitors in preclinical xenograft models for translation to clinical trials.
Specific Aim 3: To characterize the FAS expression/activity of ex-vivo surgically resected primary prostate carcinomas correlated with Gleason grade, stage, and serum levels of androgen, PSA, insulin-like growth factors I and II, and insulin-like growth factor binding protein-3.

BODY: Progress in year 1:

Much of our progress has been submitted for publication. The manuscript is provided as Appendix A, and referenced in the body of the report.

Specific Aim 1:

Task 1. Characterization of hormonal responsiveness of FAS gene and pathway: FAS expression and activity of a panel of androgen independent prostate carcinoma cells are shown in Figure 2 A and D of Appendix A. Changes in FAS enzyme content and pathway activity in response to hormonal manipulation of LNCaP and LAPC4 cell culture systems are presented in Figure 2 B,C and D of Appendix A.
Basic characterization of FAS expression in hormone responsive xenografted tumors is shown in Figure 2 E,F and G of Appendix A.

We have established transient transfection of our FAS promoter-luciferase reporter construct (1) in both LAPC4 and LNCaP, and have shown that 5α dihydrotestosterone induces promoter activity (Table 1).

Table 1

	Luc/ b-gal		Luc/ b-gal		Luc/ b-gal		Luc/b-gal
0 day DHT	1246 538		382 846		1143 1133		4888
1 day DHT	2000 1289		3444 2350		731 561		
2 day DHT	1828 2729		1324 2152		1659 1327		44926
3 day DHT	1230 1641		1881 1024		2182 1396		38600
3 day T3							8333

$5x10^4$ LAPC4 cells per well on 6 well plate, transfected with 1ug of P1, 0.5ug of pSV-beta-galactosidase control vector overnight. Treated with DHT for 0 to 3 days (Each column represents a separate experiment).

We have created the first of a planned set of mutants in the FAS promoter, which contains a deletion from -73 to –54. The deleted sequences include the major sterol response element/ E box and the insulin response element (2-4). Following sequence verification of the promoter mutant and characterization of its basal and androgen stimulated transcriptional activity, this mutant can be used to reinsert some or all missing sequences into a restriction site engineered into the deletion site.

Mutagenesis of the FAS promoter has been technically challenging due to its high GC content (80%) which limits the use of PCR based methods. High fidelity PCR mutagenesis was ultimately successful in this first mutagenesis step. Subsequent reinsertion mutants can be made without the use of PCR.

Specific Aim 2:

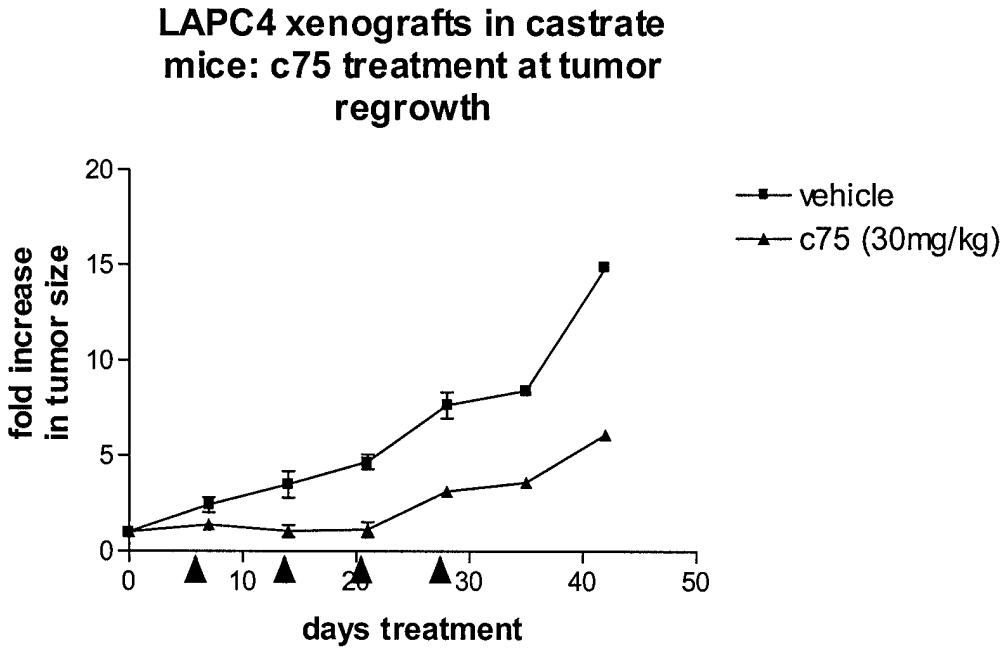
Task 1. Evaluation of FAS inhibitors, cerulenin and c75 against *in vitro* prostate cancer models.

Characterization of cytotoxicity of c75 in *in vitro* systems in parallel with hormonally manipulated changes in FAS activation is shown in Figure 3 C and D of Appendix A.

Task 2. Evaluation of FAS inhibitor c75 against xenograft prostate cancer models.

C75 treatment of hormone independent prostate carcinoma lines PPC-1 and TSU-Pr1 as xenografts is shown in Figure 4 A and B of Appendix A.

The first trial of c75 against the LAPC4 xenograft with castration of host mice and treatment of individual arms at castration, or regrowth has been performed. Treatment with c75 at castration failed due to decreased tolerance of drug toxicity. The result of treatment at regrowth is shown in the figure below. While the trend is encouraging, the small numbers of tumors that grew after castration did not provide a statistically significant data set.

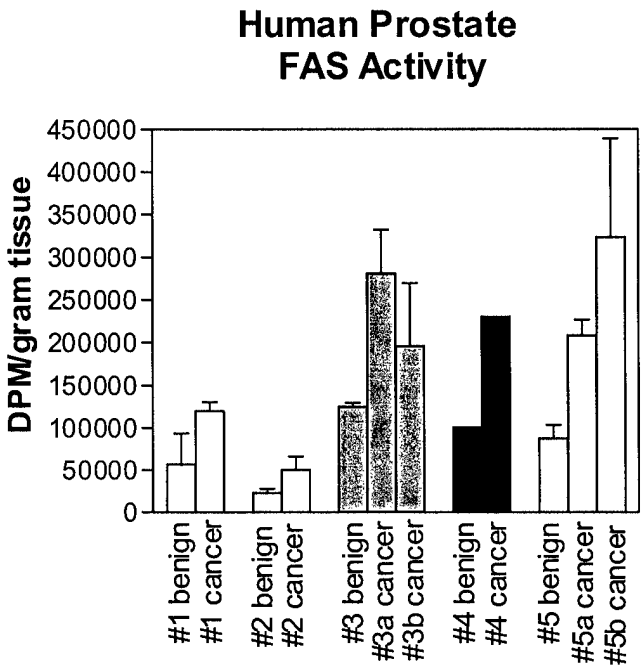


C75 inhibits the growth of LAPC4 prostate carcinoma flank xenografts in nude mice during emergent androgen independent regrowth after castration. 20 xenografted mice were castrated after establishment of flank tumors, and monitored for increases in tumor size. At regrowth, mice were randomized to treatment with weekly intra-peritoneal injections of c75 [30 mg/kg] or vehicle (arrows). Results are for 6 control and 3 c75 treated animals. There were 3 deaths in the treatment arm.

Specific Aim 3:

Task 1. Phase 1 evaluation of FAS activity in *ex vivo* prostate cancers.

Viable tumor and benign prostate tissue was harvested from 5 radical prostatectomy specimens, histologically verified, and metabolically labeled with ^{14}C-acetic acid followed by Folch extraction of lipids (5, 6). The resulting data demonstrate that fatty acid synthetic activity is consistently higher in *ex vivo* primary prostate carcinoma than in benign prostate tissue (see figure below).



Ex vivo tissues from radical prostatectomies were transferred rapidly to labeling medium after weighing. Fatty acid synthesis was assayed with a 2 hour pulse of [U-^{14}C]-acetic acid, 1μCi/ml, followed by Folch extraction and scintillation counting. All determinations were in duplicate, except when insufficient tissue was available for replicate samples. Data are presented as mean values with bars showing the standard error.

KEY RESEARCH ACCOMPLISHMENTS, YEAR 1:

- Validation of androgen responsive cell culture system and FAS promoter transient transfection assay system.
- First round mutagenesis of FAS promoter.
- Analysis of FAS expression in hormone responsive xenografts.

- Characterization of cytotoxicity of c75 in *in vitro* systems in parallel with hormonally manipulated changes in FAS activation.
- Significant growth inhibition with c75 treatment of hormone independent prostate carcinoma lines, PPC-1 and TSU-Pr1, as xenografts.
- Trial of c75 against LAPC4 xenograft with castration of host mice and treatment of individual arms at castration or at regrowth showed a trend toward growth inhibition with treatment at regrowth.
- Metabolic labeling of primary human prostate carcinoma tissue demonstrates functional activation of the fatty acid synthetic pathway in clinical disease.

REPORTABLE OUTCOMES, YEAR 1:

One manuscript has been submitted for publication, provided as Appendix A.

CONCLUSIONS:

The data generated under this project support the following conclusions: Elevated FAS expression occurs with prostate cancer progression after androgen withdrawal. In clinical practice, the progression of androgen dependent to androgen independent prostate cancer is defined by re-expression of certain gene products, most notably PSA. In this study, FAS has been identified as another potentially important gene product in prostate cancer progression. In benign prostatic epithelial cells, FAS expression was always lower than coexistent prostate cancer and, following androgen deprivation, FAS expression in benign cells was persistently reduced. In prostate cancer, however, FAS expression was elevated, both in human tissues, cells lines and xenograft models, and escaped the effects of androgen withdrawal. Therefore, like PSA, although FAS initially responded to the androgenic environment, the re-expression of FAS was also characteristic of the ultimately fatal, androgen independent disease.

Elevated FAS expression is a marker for elevated fatty acid synthesis. As supported by the [U ^{14}C] acetate labeling data in cell lines, xenograft tissue, and human tumor tissue, elevated FAS expression serves as a marker for a highly active metabolic pathway in cells. Each of these tumor samples with high FAS had elevated fatty acid synthesis relative to control lines and tissues. Expression of this phenotype requires coordinate regulation of numerous cellular functions to produce the substrates for fatty acid synthesis, and to maintain an anabolic state. The frequent emergence of this complex phenotype from androgenic regulation during tumor progression suggests a functional role for active fatty acid synthesis in the growth or survival of prostate carcinoma cells. The cell culture systems and FAS promoter-luciferase reporter plasmids generated during the first year of the project provide the tools to discover the regulatory mechanisms involved.

FAS inhibition is therapeutic *in vivo* against prostate carcinoma xenografts. This tumor progression associated function provides a novel therapeutic target for androgen independent prostate cancer. In this study, we demonstrated a significant growth inhibition of two human, androgen independent tumors following systemic administration of the FAS inhibitor, c75, in doses that were well tolerated by the host animals. This

result indicates that the FAS activation typical of late stage, androgen independent prostate cancer may confer clinically useful sensitivity to FAS anti-metabolites.

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Appendix A

Increased Fatty Acid Synthase as a Therapeutic Target in Androgen Independent Prostate Cancer Progression

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Abstract

Background: Fatty acid synthase (FAS) performs the anabolic conversion of dietary carbohydrate or protein to fat. FAS expression is low in most normal tissues, but is elevated in many human cancers, including androgen sensitive and androgen independent prostate cancer. **Methods:** Immunohistochemical evaluation of FAS expression was performed in human prostate cancer specimens under various states of androgen ablation. *In vitro* and *in vivo* prostate cancer models were evaluated for FAS expression and activity under androgenic and androgen depleted conditions, and were tested for sensitivity to anti-metabolite drugs that target fatty acid synthesis. **Results:** While FAS expression in the prostate was androgen responsive, it persisted or was reactivated in human prostate carcinoma after androgen ablation, and was high in 82% of lethal tumors examined at autopsy. Similar patterns of FAS expression and fatty acid synthesis were seen in cell culture and xenograft models of human prostate cancer. Pharmacologic inhibition of FAS resulted in a dose-dependent reduction of tumor growth in these models, including four-fold inhibition of an androgen independent human prostate cancer xenograft with little associated toxicity. **Conclusions:** The data suggest that FAS expression/FA synthesis provides an important functional aspect of the malignant phenotype in prostate cancer, perhaps supporting cell growth or survival. FAS expression may be upregulated by alternate signaling pathways important for prostate cancer growth under androgen withdrawal. The reemergence of FAS expression and activity during the development of androgen independence demonstrate that FAS may serve as a novel target for anti-metabolite therapy in prostate cancer.

Introduction

In prostate cancer, treatment with androgen ablation often produces dramatic objective responses, yet from this initial hormone sensitivity emerges lethal prostate cancer, an androgen independent disease that kills almost 40,000 men a year in the United States. Certain androgen-regulated gene products, such as the biomarker prostate specific antigen (PSA), will predictably decline after androgen ablation, but a subsequent rise in PSA heralds the advent of androgen independent disease progression. Identifying and targeting other gene products uncoupled from androgen regulation is an attractive therapeutic approach. One such candidate gene product is the biosynthetic enzyme, fatty acid synthase (FAS), which, like PSA, shows increased gene expression and enzyme activity during androgen stimulation of androgen responsive prostate carcinoma cells [1, 2]. FAS is the major enzyme required for the anabolic conversion of dietary carbohydrate to fatty acids. It functions normally in the liver to make lipids for export to metabolically active tissues or storage in adipose tissue [3]. FAS has specialized physiological functions producing milk lipids in lactating breast tissue, surfactant in lung, and may contribute to the production of ejaculate lipids by the prostate [4-7]. However, FAS expression is low in most normal adult tissues, which appear to utilize circulating lipids preferentially for the synthesis of new structural lipids [8]. FAS is expressed at markedly elevated levels in biologically aggressive subsets of human prostate cancers, as well as breast and other cancers [9-13], and cell lines derived from these tumors display concordant elevation of FAS enzyme content and fatty acid synthetic activity [14]. Similarly, *ex vivo* colorectal carcinoma tissue displays elevated FA synthesis that parallels FAS expression levels compared to adjacent non-neoplastic mucosa [15]. While

the malignancy-associated function of FAS is not yet determined, the major lipid product in most tumors is phospholipid. Likewise, the mechanism(s) for up-regulation of FAS in tumor cells is unknown. FAS expression likely provides an important function for tumor cells, since FAS upregulation is common and linked to tumor virulence in studies of human tissues. Furthermore, FAS inhibitors are selectively cytotoxic to tumor cells in experimental systems, apparently due to acute intracellular accumulation of the committed substrate, malonyl-CoA, that only occurs when fatty acid synthesis is active [16-18].

These observations prompted the present study of FAS expression and activity in human prostate cancer, with the hypothesis that the initial sensitivity of FA synthesis to androgen withdrawal in prostate cancer is lost in parallel with the emergence of the androgen independent phenotype, and that specific inhibition of FAS thus provides a therapeutic target for prostate cancer. We therefore studied the expression of FAS in matched benign and malignant prostatic tissues and cell lines under different hormonal conditions, and in metastatic lesions obtained at autopsy from men dying of androgen independent disease, and established the sensitivity of prostate cancer cell lines to FAS inhibition *in vitro* and *in vivo*.

Materials and Methods

Prostate carcinomas. Radical prostatectomy specimens with tumors of intermediate or high grade were obtained from the surgical pathology files of The Johns Hopkins Hospital and The Johns Hopkins Bayview Medical Center. Autopsy tissues were obtained

as part of the Project to ELIminate lethal CANcer (PELICAN) at Johns Hopkins University School of Medicine.

Immunohistochemistry. 4 or 5-μm sections were subjected to heat induced epitope retrieval, followed by monoclonal anti-human-FAS antibody at a concentration of 0.1 to 0.5 μg/mL (U.S. patent number 5,864,011), detected with the LSAB2 system from DAKO.

Immunohistochemical interpretation. Analysis of immunostaining for FAS was performed using a scoring system based on summation of negative, weakly positive and strongly positive staining in each tissue, with FAS expression values assigned on a scale of 0 to 8, as described [11]. Graphing and statistical analysis were performed in SigmaPlot 5.0 (SPSS).

Cell lines and culture conditions: With the following exceptions, cell lines were obtained from the American Type Culture Collection, and were cultured in RPMI-1640 with 10% fetal bovine serum (Hyclone). TSU-Pr1 cells [19] PPC-1 cells [20] and LAPC-4 cells [21] were provided by the originating laboratories. The LAPC-4 cell line was grown in Iscove's Medium with 10% fetal bovine serum. The androgen deprived clones of LNCaP cells were derived as previously described [22] and were grown continuously in RPMI-1640 (without phenol red) with 10% charcoal stripped fetal bovine serum (Hyclone). The LAPC-4 AD cells were androgen deprived 4-6 months in Iscove's Medium (without phenol red) with 10% charcoal stripped FBS. Normal prostate epithelial cells (PrEC) and stromal cells (hPS) were purchased from Biowhittaker (Clonetics). The hPS cells were grown in RPMI-1640 with 10% FBS and the PrEC cells were grown in PrEGM (Biowhittaker) supplemented according to the manufacturers

recommendations. Cells were plated in 24 well culture plates at 5 x 10^4 cells/well 24 hours before treatment. Cerulenin (Sigma) and c75 were added as stock 5mg/ml solutions in DMSO. The final concentration of DMSO in cultures was at or below 0.2%.

Immunoblot analysis: Cells were lysed in 10 mM Tris buffer (pH 8.0) containing 135 mM NaCl, 1% TritonX-100, 10% glycerol with antipain (5 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (1 μg/ml), phenylmethylsulfonyl fluoride (1mM) added fresh. The lysates were centrifuged at 12,000 x g for 10 min and the supernatants used for immunoblot analysis. Equal amounts of protein (20 μg of cell line and xenograft, 80 μg of mouse liver) were separated on 4% SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes. Immunodetection was with rabbit anti-FAS antibody (1:4000) [14] followed by secondary antibody (goat anti-rabbit HRP; 1:20,000, Boehringer Mannheim). After washing, immunoreactivity was visualized using ECL (Amersham) and Kodak XAR film.

Metabolic labeling: Cultured cells were plated at 5x10^4/well in 1 ml in 24 well plates and incubated overnight prior to labeling. Replicate cultures were trypsin treated and counted to correct for plating efficiency and growth. *Ex vivo* tissues from xenografted mice were transferred rapidly to labeling medium after weighing. Fatty acid synthesis was assayed with a 2 hour pulse of [U-^{14}C]-acetic acid, 1μCi/ml, followed by Folch extraction and thin layer chromatography with standards, as described [16]. Quantitation of lipid classes was performed on a Storm (Molecular Dynamics). All determinations were in duplicate, except when insufficient xenograft tumor was available for replicate samples. Data are presented as mean values with bars showing the standard error. Calculations and graphing were performed in Prism 2.0 (GraphPad).

Xenograft studies: Subcutaneous flank xenografts of the human prostate cancer cell lines, LAPC-4 and PPC-1 were established from cells in culture in 4-6 week old, male nu/nu mice (National Cancer Institute). All animal experiments complied with institutional animal care guidelines. Mice received subcutaneous flank injections of 4 x 10^6 LAPC-4 cells in Iscove's Medium, and, after two months, the animals with tumors were randomized to castration (n=10) or left intact (n=3). Upon regrowth of the tumors, the animals were sacrificed and FAS expression and activity of each xenograft was analyzed as described above.

Other mice were injected with 2 x 10^6 PPC-1 or TSU-Pr1 cells in RPMI-1640, and after tumors were palpable, animals were randomized to treatment with weekly doses of C75 at 30 mg/kg/ip, or 3 mg/kg/ip, or vehicle (20% DMSO/ 80% RPMI/ip) (n=10 animals/arm). Tumor volume was measured with calipers in three dimensions. The experiments were terminated when the controls reached the surrogate endpoint.

Results

FAS expression in prostate cancer tissues: Thirty one intermediate and high grade primary prostate cancer specimens were evaluated by FAS immunohistochemistry, and expression was scored by a combined assessment of FAS levels and distribution [11] (Figure 1). Twenty one were from intact and 10 were from androgen ablated patients. The benign epithelium from hormonally intact patients displayed widely variable FAS immunoreactivity, both within and among individual samples. In all but 2 cases, the tumors in these prostates expressed more FAS than the benign tissue. In the 10 cases studied after three to six months of androgen deprivation therapy, FAS expression was

significantly reduced in the benign epithelium relative to the intact group (Mean FAS score 2.4 versus 4.8, Student t test, T=5.84, p=0.000002). However, FAS expression in the tumors remained comparable to control levels even after androgen ablation (Mean FAS scores 7.0 and 7.1). Similarly, when 77 samples of primary and metastatic prostate carcinoma were evaluated from 18 autopsy cases, all of which had been treated with prolonged androgen ablation, elevated FAS immunoreactivity (FAS score $\geq$4) was found in 82%, while the residual benign prostatic epithelium had very low FAS. Thus, while FAS expression was androgen responsive in benign prostatic epithelium, it persisted following androgen ablation in the remaining viable tumor, and showed high expression in lethal, androgen-independent disease.

FAS in model systems of prostate cancer: *In vitro* and *in vivo* models of human prostate cancer provide the opportunity to characterize FA synthesis pathway function in parallel with FAS expression, and to manipulate tumor growth conditions in a controlled manner. The steady state expression levels of FAS and FA synthesis were characterized in 11 prostate carcinoma cell lines, including 4 androgen independent lines, 2 androgen dependent lines, and their 5 androgen independent subclones, selected after chronic growth in androgen depleted media (Figure 2A and B). FAS enzyme content was higher in the androgen independent tumor lines than in benign prostatic epithelial and stromal cells (Figure 2A), and was very high in the two androgen dependent lines, LNCaP and LAPC4 (Figure 2B). Comparison of LNCaP and LAPC4 with their subclones grown in androgen depleted conditions demonstrated substantial reduction in FAS protein content after androgen withdrawal, consistent with androgen stimulation of FAS expression. These androgen deprived clones retain the ability to respond to androgen, and

reintroduction of dihydrotestosterone to one of these lines partially restored FAS expression (Figure 2C). Most of the tumor lines also had elevated FA synthetic pathway activity compared to benign prostatic epithelial and stromal cells, although LAPC4 did not (Figure 2D). Although the major product of the fatty acid synthesis pathway in these cell lines was membrane phospholipid, substantial amounts of triglyceride were also produced. Other lipid classes represented less than 10% of the total extractable lipids, and are not shown. The very high FA synthetic activity of LNCaP was substantially reduced in the androgen deprived subclones, LN95-98 (Figure 2D), similar to enzyme content (Figure 2B).

FAS phenotype in an androgen dependent prostate carcinoma xenograft model: The LAPC4 prostate carcinoma line produces androgen sensitive subcutaneous flank tumors in male nu/nu mice: following initial regression after castration and prolonged latency, the tumors begin to grow again, providing a model of chronic androgen deprivation and, ultimately, androgen independence [21, 23]. We determined the expression levels of FAS and measured FA synthesis *ex vivo* in a cohort of LAPC4 xenografts that had re-grown 6 months after castration of the host animals. FAS expression and function were both elevated in all xenografts examined during re-growth (Figure 2E and F), by comparison with liver (Figure 2E and G). The cases illustrated are representative of the cohort. While some xenografts from castrated animals showed reduced FAS content relative to tumors from intact animals (xenograft 8), others showed no reduction in FAS content (xenograft 10). Tumor FA synthesis activity was comparable in castrated and intact animals, and an order of magnitude higher than in liver. Thus, during emergent androgen independent growth, FAS expression was high, generating a

phenotype similar to the androgen independent cell lines, DU145, PC3, PPC-1 and TSU-Pr1. Indeed, *ex vivo* pulse labeling of PPC-1 xenografts demonstrated similar FA synthetic activity (data not shown).

Sensitivity of prostate cancer models to FAS inhibitors: Studies of experimental models of other cancers with high FAS have shown sensitivity to FAS inhibitors [14, 16-18]. Most of these utilized the anti-metabolite, cerulenin, a natural product of the fungus, *Cephalosporium caerulens*. Cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-trans,trans-dodecadienamide, has been known since the 1960s as a specific inhibitor of FA synthesis across a broad phylogenetic spectrum [24]. Cerulenin irreversibly inhibits FAS by formation of a covalent adduct with the active site cysteine of the beta keto acyl synthase moiety, which performs the condensation reaction between the elongating fatty acid chain and each successive malonyl residue. We have recently developed a novel small molecule inhibitor of FAS, called c75, with comparable inhibitory effects on FA synthesis and greater chemical stability than cerulenin [25]. Exposure of human cancer cells to cerulenin or c75 triggers apoptosis [17, 18]. To evaluate the sensitivity of prostate cancer cells to FAS inhibitors, and to determine whether androgenic modulation of FAS in tumor cells would alter their sensitivity to these drugs, we evaluated the growth inhibition in response to anti-metabolites cerulenin or c75 of 4 androgen independent human prostate cancer cell lines, 2 androgen dependent lines and their 5 androgen depleted subclones (Figure 3). Both cerulenin and c75 demonstrated selective, dose-dependent cytotoxicity for tumor cells, with IC_{50}s in the dose range of 2 to 8 micrograms/ml and 1 to 4 micrograms/ml respectively. Benign prostatic epithelial and stromal cells (PrEC and hPS in Figure 3) were significantly less sensitive to these agents

at several doses levels. Although the androgen sensitive lines LNCaP and LAPC4 had comparable sensitivities, their androgen independent subclones were significantly less sensitive to cerulenin, and the LNCaP subclones showed modestly decreased sensitivity to c75, in parallel with the decreased FAS expression and FA synthesis demonstrated in Figure 2. In separate experiments, FAS inhibitors induced apoptosis in these cell lines (data not shown).

Sensitivity of prostate cancer xenografts to FAS inhibition: Given the potency of the FAS inhibitors against prostate cancer cell lines in vitro, the therapeutic potential of c75 was studied using subcutaneous xenografts of the androgen independent, human prostate cancer cell lines PPC-1 and TSU-Pr1 in nu/nu mice. Cerulenin has limited utility for *in vivo* FAS inhibition studies because it is chemically unstable, and does not produce systemic activity. The pharmacological properties of c75 are substantially improved in these regards. Cohorts of 10 male nude mice carrying established, palpable flank xenografts of PPC-1 were treated with 4 intraperitoneal doses of c75 on a weekly schedule (Figure 4A). Mice treated with high dose c75 (30 mg/kg/week) had a greater than 4-fold reduction in tumor growth, relative to low dose c75 (3 mg/kg/week) and vehicle treated control mice. A similar treatment protocol (omitting the low dose c75 arm) produced 2.7-fold growth inhibition of TSU-Pr1 flank xenografts (Figure 4B).

The FAS inhibitors produce a transient weight loss resulting from appetite suppression related to malonyl Co-A accumulation in the brain [14, 26]. C75 toxicity was further evaluated in mice without tumors using the same high dose and schedule (30 mg/kg/week x 4 injections), followed by necropsy with gross and microscopic pathologic survey of organs and tissues. In parallel with the observed weight loss, c75 treated mice

demonstrated lipid depletion from adipose tissue (Figure 4 C and D). No other consistent organ damage was observed.

Discussion

Elevated FAS expression indicates progression after androgen withdrawal. In clinical practice, the progression of androgen dependent to androgen independent prostate cancer is defined by re-expression of certain gene products, most notably PSA. In this study, FAS has been identified as another potentially important gene product in prostate cancer progression. In benign prostatic epithelial cells, FAS expression was always lower than coexistent prostate cancer and, following androgen deprivation, FAS expression in benign cells was persistently reduced. In prostate cancer, however, FAS expression was elevated, both in human tissues, cells lines and xenograft models, and escaped the effects of androgen withdrawal. Therefore, like PSA, although FAS initially responded to the androgenic environment, the re-expression of FAS was also characteristic of the ultimately fatal, androgen independent disease.

Previous studies of FAS expression in primary prostate carcinomas demonstrated variable FAS expression levels in intermediate grade tumors that were strongly predictive of pathologic stage or recurrence [11, 12]. In these studies, benign prostatic glands were either negative or only weakly immunoreactive with antibody to FAS. Recent advances in immunohistochemistry, including availability of a monoclonal antibody against human FAS, have allowed increased sensitivity of detection of FAS in tissue sections. Similar FAS immunoreactivity of benign prostatic glands has recently been reported by another group, using an anti-peptide antibody to FAS [27].

Elevated FAS expression marks a cellular function. As supported by the [U ^{14}C] acetate labeling data, elevated FAS expression serves as a marker for a highly active metabolic pathway in cells. Prostate carcinoma tumor lines and xenografts with high FAS had elevated fatty acid synthesis relative to control lines and tissues. Phospholipids and triglycerides represented the bulk of the lipid products of the pathway. Expression of this phenotype requires coordinate regulation of numerous cellular functions to produce the substrates for fatty acid synthesis, and to maintain an anabolic state. The frequent emergence of this complex phenotype from androgenic regulation during tumor progression suggests a functional role for active fatty acid synthesis in the growth or survival of prostate carcinoma cells. This idea is also supported by the association of FAS expression with poor prognosis in other studies of prostate cancer, and in cancers of other organ systems [9-13].

Inhibition of vital cellular functions often produces cytotoxicity, as demonstrated by targeted blockade of fatty acid synthesis in prostate cancer models. The FAS inhibitors, cerulenin and c75, were cytotoxic to prostate carcinoma lines, with greater sensitivity in tumor lines that had high FAS and fatty acid synthesis relative to controls, or tumor lines with down regulated FAS.

FAS inhibition is therapeutic *in vivo* against prostate carcinoma xenografts. The identification of fatty acid synthesis reactivation during tumor progression raises new questions about the cell biology of prostate cancer. Perhaps more important is the identification of a novel therapeutic target for androgen independent prostate cancer. In this study, we demonstrated a significant growth inhibition of two human, androgen

independent tumors following systemic administration of the FAS inhibitor, c75, in doses that were well tolerated by the host animals. This result indicates that the FAS activation typical of late stage, androgen independent prostate cancer may confer clinically useful sensitivity to FAS anti-metabolites.

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Figure Legends

Figure 1. FAS immunoreactivity of prostate cancer tissues. A and B. Sections from radical prostatectomies from patients without and with prior androgen ablation therapy. Benign glands (arrows) show moderate (A), or very low (B) FAS expression. Tumor (all unlabeled glands) has uniformly high FAS. **C and D.** Sections from bony metastasis and liver metastasis of prostate cancer sampled at autopsy. Tumor cells show high FAS expression in contrast to negative expression in bone marrow and low expression in liver. **E.** Scatter plot of FAS immunoreactivity scores of benign glands (o) and tumor (•) for radical prostatectomies from 21 intact and 10 androgen ablated patients, and from prostates of 9 patients with lethal prostate cancer sampled at autopsy after prolonged androgen ablation. Significant reduction in mean FAS expression score resulted for benign epithelium after androgen ablation (Student t test, $p=0.000002$), while tumor scores were not significantly different. **F.** Scatter plot of FAS immunoreactivity scores of 68 metastatic deposits of prostate cancer from 18 autopsies.

Figure 2. Fatty acid synthase activation in prostate carcinoma cell lines and a prostate carcinoma xenograft under emergent androgen independence.

A and B. FAS enzyme levels in prostate cancer cell lines detected by immunoblot. 20 micrograms of protein were loaded in each lane. **A.** DU145, PC3, PPC-1 and TSU are androgen independent tumor lines. PrEC and hPS are benign epithelial and stromal cells, respectively. **B.** LAPC4 and LNCaP are androgen dependent tumor lines. LAPC-AD is a subclone of LAPC4 selected in androgen depleted culture. LN95-98 are subclones of LNCaP selected in androgen depleted culture. These clones, though androgen independent, remain androgen sensitive. **C.** LN96 cells were cultured under control conditions, or were exposed to 5 alpha-dihydrotestosterone [10^{-9} M] for 1 to 7 days before immunoblot analysis of FAS content. 20 micrograms of protein were loaded in each lane. The parental line, LNCaP is shown for comparison. **D.** Fatty acid synthesis activity and products of these tumor lines. Samples were pulse labeled with ^{14}C-acetate, lipids were extracted and separated by thin layer chromatography into major classes. Minor lipids representing less than 10% of total not shown. **E.** FAS enzyme levels in tissues from xenografted mice detected by immunoblot. LAPC4 xenografts: Animals 8 and10 were castrated. Animals 11 and 12 were left intact. Tumor lysates, 20 micrograms; liver lysates, 80 micrograms. Immunohistochemical evaluation of the xenografts demonsrated similar staining to the human tissue samples in Figure 1 (not shown). **F and G.** Fatty acid synthesis activity and products of these tissues. Fatty acid synthesis was an order of magnitude more active in the tumors than in liver (scale is different for F and G). Tumor lines and xenografts show elevation of both FAS expression and FA synthesis

over controls. Xenografts studied during periods of active growth have elevated FAS expression and FA synthesis without or with androgen ablation.

Figure 3. Growth Inhibition of Prostate Carcinoma lines by FAS anti-metabolites. **(A and B)** FAS anti-metabolites, cerulenin and c75, inhibit the growth of androgen independent prostate carcinoma lines DU145, PC3, PPC-1, and TSU with IC_{50}s in the range of 2 to 8μg/ml and 1 to 4μg/ml respectively. Non-transformed lines, PrEC and hPS are less sensitive. **(C and D)** Prostate carcinoma lines LAPC4 and LNCaP, with high FAS under androgen stimulation, show greater growth inhibition by cerulenin than their low FAS expressing subclones selected in androgen depleted culture. Changes in sensitivity to c75 were minimal.

Figure 4. C75 inhibits the growth of prostate carcinoma flank xenografts in nude mice. PPC-1 flank xenografts **A.** TSU-Pr1 flank xenografts **B.** 10 xenografted mice per cohort were treated with weekly intra-peritoneal injections of c75 or vehicle (arrows). C75 dosed at 30 mg/kg produced four-fold inhibition of PPC-1 tumor growth relative to controls and low dose c75, and 2.7-fold inhibition of TSU-Pr1 tumor growth. **C.** control and **D.** c75 treated mouse bowel. In a parallel experiment, c75 dosed at 30 mg/kg produced marked depletion of lipid from adipose tissues, but other organs and tissues showed no histologic changes after c75.

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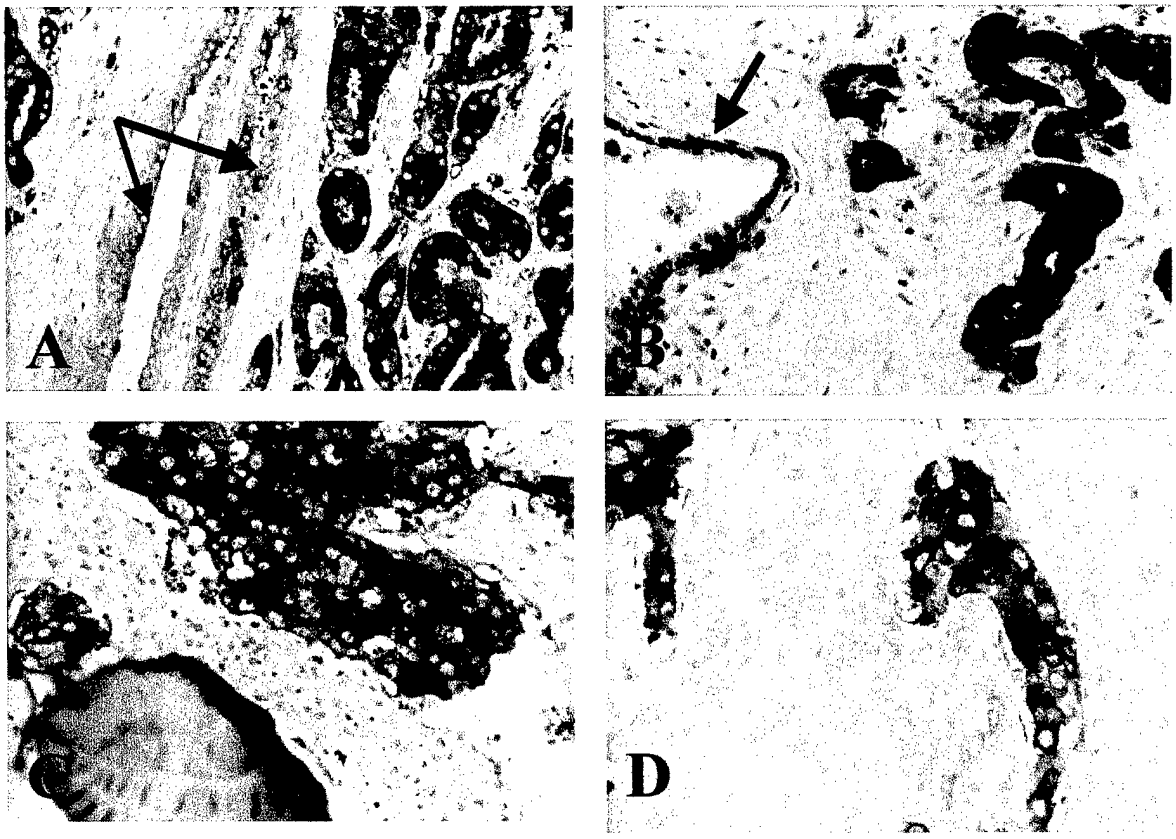
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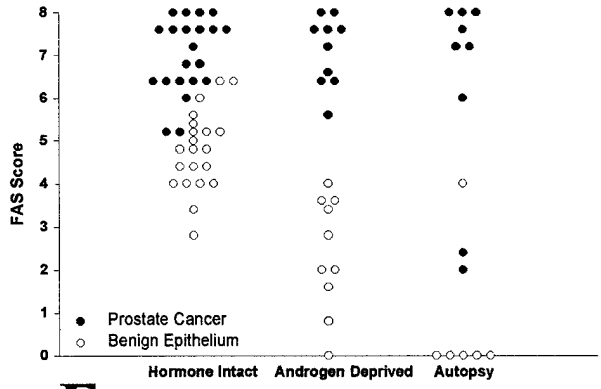
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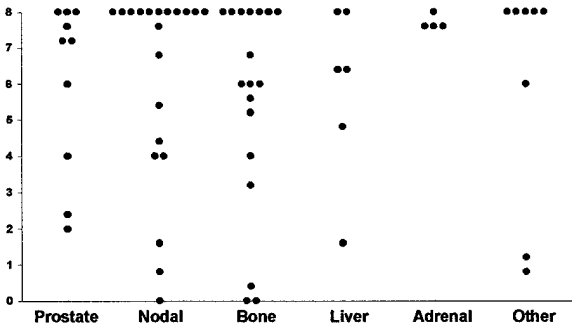
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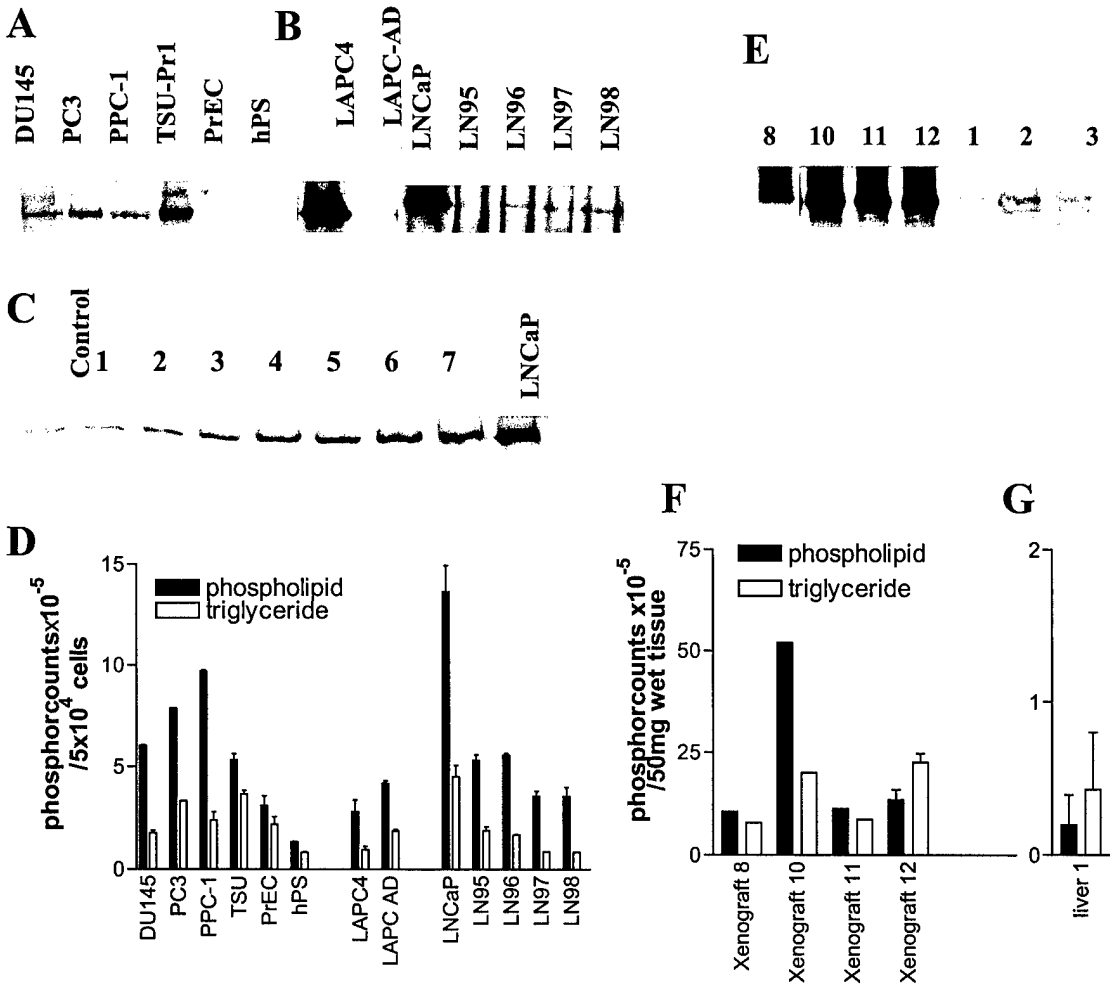
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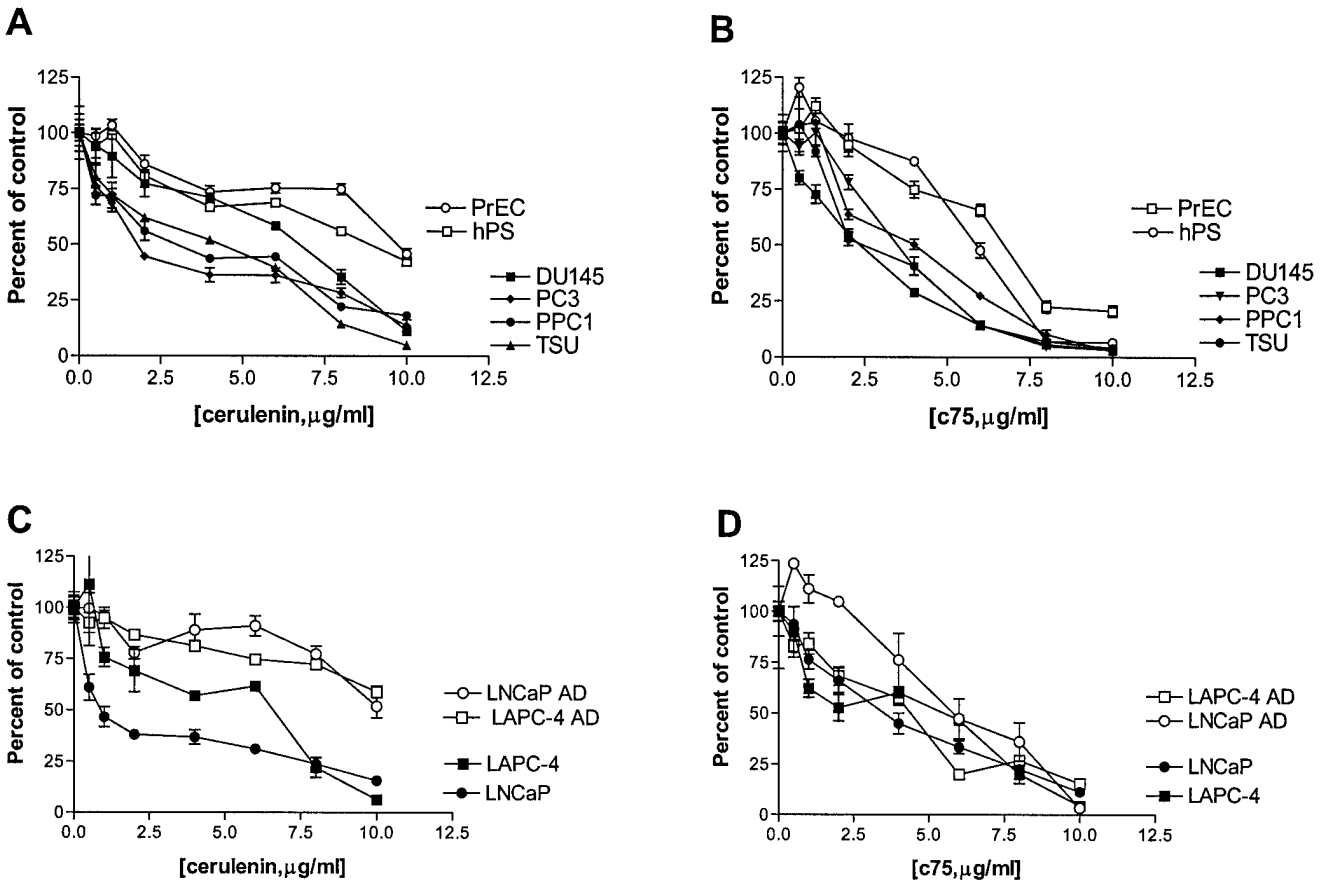
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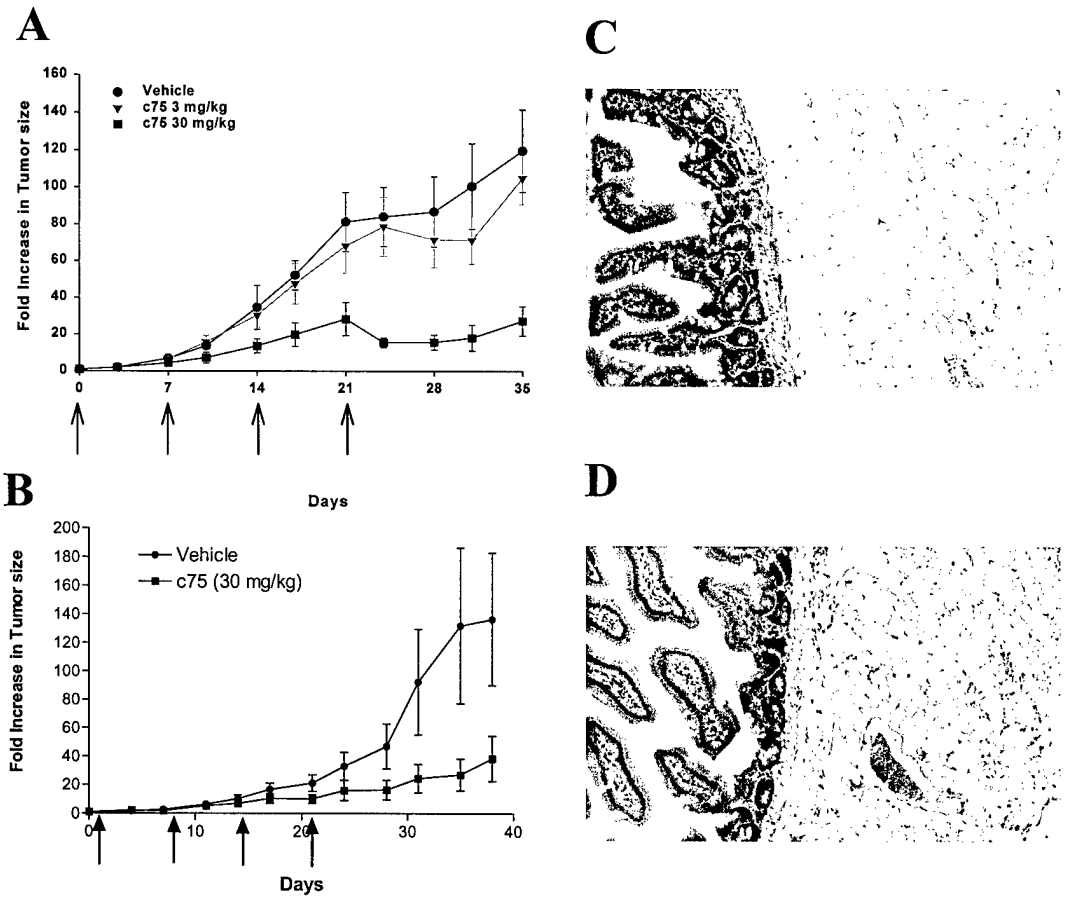
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c75 (30 mg/kg)
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