AWARD NUMBER: DAMD17-03-1-0522

TITLE: Constitutive Activation of NF-κB in Prostate Carcinoma Cells Through a Positive Feedback Loop: Implication of Inducible IKK-Related Kinase (IKKi)

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REPORT DATE: August 2005

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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Constitutive Activation of NF-κB in Prostate Carcinoma Cells Through a Positive Feedback Loop: Implication of Inducible IKK-Related Kinase (IKKi)

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The overall goal of this project is to understand the role of inducible IKK-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells. During FY02 we developed the conditions for RNA isolation from OCT-embedded frozen PC and BPH samples, developed conditions for cell lysis and IKKi immunoprecipitation from transfected cells using FLAG antibody. We also generated PC cell lines stably infected with lentiviruses harboring w.t. IKKi, d.n. IKKi mutant K38A, and constitutively active IKKi mutant S172E. We continued the characterization of biological effects of IKKi overexpression/IKKi blockage in PC cells. Our data provide the experimental evidence that IKKi could be involved in the regulation of NF-kB activity in PC cells through a positive feedback loop. Inactivation of IKKi in PC cells via infection with lentivirus expressing IKKi d.n. mutant indicated that IKKi plays an important role in the regulation not only NF-kB but also other transcriptional factors. IKKi appeared to be very important for PC cell growth regulation. The in vitro growth of PC3 cells infected with IKKi d.n. mutant was reduced by 60%. The results of our studies have been presented at the local and national meetings, two manuscripts are published, one is in press, and the fourth manuscript is under preparation.
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Introduction

The overall goal of this project is to understand the role of inducible IKK-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells.

During FY02 the major directions of our work were: i) to study the co-localization of IKKi and its substrate TRAF2 in PC samples, ii) to develop the conditions for RNA isolation from OCT-embedded frozen PC and BPH samples; iii) to develop mild conditions for cell lysis and IKKi immunoprecipitation using FLAG antibody; iv) to generate PC cell lines stably infected with lentiviruses harboring w.t. IKKi, d.n. IKKi mutant K38A, and constitutively active IKKi mutant S172E; v) to continue characterization of biological effects of IKKi overexpression/IKKi blockage in PC cells.

During summers 2004 and 2005 my laboratory participated in summer student training program, and this year one of the summer students was involved in the studies focused on the role of NF-kB and upstream kinases in prostate tumorigenesis funded by DOD. The results of our studies have been presented at the local and national meetings, two manuscripts are published, one is in press, and another manuscript is under preparation. The following describes the progress made in this year.

Body

During current year we specifically focused on the experiments pertinent to the tasks 2 and 3.

Task 2.

Using prostate samples provided by NU prostate SPORE tissue core we performed immunostaining of more than 60 formalin-fixed samples of BPH, PIN, and PCs using multiple antibodies against IKKi (four different Abs from Imgenix, Santa Cruz., Active Motif, Pro-Sci). The analysis of IKKi staining in prostate tissue samples indicated that IKKi had nuclear localization and was highly expressed in prostate glands, but not in prostate stromal fibroblasts. We also performed analysis of TRAF2 expression and localization in BPH and PC samples using Santa Cruz antibodies against this IKKi
substrate. Analysis of expression performed by our summer student Meera Sekar, has not revealed the significant correlation between IKKi and TRAF2 localization in PCs.

The initial Northern blot analysis of IKKi and its substrates has not revealed any differences in gene expression between BPH and PC samples. However, we used for this pilot analysis snap frozen PC samples with low percent (20-25%) of tumor cells obtained via Cooperative Human Tissue Network. Currently we are using OCT-embedded frozen PC samples with high percent of tumor cells (up to 50-70%) provided by NU prostate SPORE tissue core. The RNA extraction from OCT-embedded samples required the modification of the standard protocol. We have developed SOP, and successfully tested the modified protocol for RNA isolation.

**Task 3.**

We have generated several independent clones of LNCaP and PC3 cell lines stably expressing IKKi w.t. and IKKi d.n. mutant. The stable lines were generated using commercially available lentiviral system (Invitrogen Corporation) with the expression of IKKi and its mutants driven by CMV promoter. The lentiviral technology allowed us to make several improvements for stable transfections of PC cells such as: i) the increased number (up to 85-90%) of IKKi-expressing cells in the bulk culture; ii) long term stability of exogenous IKKi expression, and iii) to achieve physiological levels of IKKi expression comparable to the endogenous level of expression. We have prepared the review paper published at R. Lurie Comprehensive Cancer Center Journal on the technical aspects of PC cell infection with lentiviruses, we also actively consulting other researchers at NU how to use the optimized conditions for lentiviral infection of epithelial cells.

In PC infected cell cultures both w.t. and mutant forms of IKKi were tagged with FLAG for easier detection in vitro and in vivo and for immunoprecipitation. We have successfully precipitated IKKi w.t. and IKKi d.n. from stably transfected LNCaP cells using anti-FLAG antibodies conjugated to agarose (Sigma). We have also established mild procedure for cell lysis and IKKi immunoprecipitation to ensure the stability of IKKi–protein complexes. Currently, we are analyzing IKKi-interacting proteins by western blotting. In FY03 we plan to continue analysis of the IKKi-interacting proteins using 2-D gel electrophoresis following Mass-Spectroscopy analysis of differentially expressed protein spots at Northwestern University Biotechnology and Proteomics Core Facility (Evanston, IL).

We have already made one of the additional proposed IKKi mutants (S172E). It
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is a constitutively active kinase that will allow us to test the hypothesis that nuclear localization depends on IKKi activation. We are in the process of subcloning S172E mutant into lentivirus expressing vector that will be used in FY03 for generation of stably infected PC cell lines with constitutively active IKKi. To expand the search for IKKi targets and affected signaling pathways, besides NF-κB, we have studied IKKi effect on a few transcription factors critical for PC development such as STAT-1, STAT-3, and AP1 using transient transfections with the appropriate Luciferase reporters. We found that IKKi d.n. dramatically inhibited both basal and inducible activity of NF-κB and AP-1 transcription factors. IKKi d.n. also inhibited STAT1 and STAT3 activity, however its effect was less pronounced. These data suggest that IKKi may be involved in the regulation of a number of signaling pathways and may have multiple phosphorylation targets.

We also started to study the major biological outcomes (including the apoptosis and growth) of IKKi overexpression/ablation in PC cell cultures. We found that expression IKKi d.n. in PC3 abolished their proliferation by 60-70% as compared to PC3-empty vector infected cells.

Finally, taking into consideration the newly recognized association of prostate inflammation and prostate cancer that offers one of the greatest opportunities for preventing malignant conversion (De Marzo et al., 2003, Platz and De Marzo, 2004) we dedicated some additional effort to study the cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor in prostate carcinoma cells. We found that glucocorticoids inhibit IKKi function, and that GR acts at early stages of prostate tumorigenesis as a tumor suppressor.

Key Research Accomplishments

- We found that IKKi is expressed in glandular component of prostate samples. Nuclear IKKi expression was increased in human prostate carcinomas in comparison to BPH samples. Image analysis did not reveal correlation between IKKi and TRAF2 expression in the nuclei of PC cells.

- We have developed protocol for RNA isolation from OCT-frozen prostate samples.
We have developed protocol for optimal PC cell stable infection by lentiviruses. We have prepared and published at the journal of R. Lurie Cancer Center Journal the technical review and the manual on the preparation of high titer viral stocks and the epithelial cell infections by lentiviruses.

We have generated several lentiviruses harboring empty vector, IKKi-FLAG, d.n. IKKi-FLAG, and generated stably infected PC3 and LNCaP cell lines.

We found that d.n. IKKi negatively affected several transcription factors in PC cells including NF-kB, AP1, and STAT factors.

We showed that the expression of IKKi d.n. mutant in PC3 significantly reduced their growth in vitro.

These data provide experimental evidence that IKKi could be involved in the regulation of different transcription factors besides NF-kB, and is important for PC cell growth.

Reportable outcomes:

Abstracts:


Presentations by P.I.:


2. Effect of NF-kB inhibitor PS1145 and glucocorticoids on prostate carcinoma cells. Prostate SPORE, R. Lurie Cancer Center, Northwestern University, Chicago, IL, September 2004.

3. Targeting NF-kB transcription factor and IKK kinases in prostate carcinoma cells. The University of Auckland, School of Medicine-Auckland Cancer Society Research Center, Auckland, New Zealand, November 2004.

4. Targeting the transcription factor NF-kB for intervention of prostate and skin cancer. Ludwig Institute for Cancer Research and Royal Melbourne Hospital, Melbourne, Australia, November 2004.

5. Constitutively active NF-κB transcription factor and IKKb kinase in human prostate carcinoma cells as a possible targets for intervention. Epithelial group seminar series.

3. Publications.


Conclusions

Our data provide experimental evidence that IKKi could be involved in the regulation of NF-kB activity in PC cells through a positive feedback loop. Inactivation of IKKi in PC cells via infection with lentivirus expressing IKKi d.n. mutant indicated that IKKi plays an important role in the regulation of NF-kB and other transcriptional factors. IKKi also is very important for PC cell growth regulation.

References


Appendices


Effects of IKK inhibitor PS1145 on NF-κB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells.

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Running title: Effect of IKK inhibitor on prostate carcinoma cells

Key words: prostate carcinoma cells, NF-κB, IKK, inhibitor, apoptosis, proliferation, invasion

Abbreviations:
EMSA – electrophoretic mobility shift assay; Erk - Extracellular signal-regulated kinase; IAP – inhibitor of apoptosis; IKK - Inhibitor of nuclear factor kappa-B kinase; IκB - Inhibitor of nuclear factor kappa-B; IL – interleukin; LPS – lipopolysaccharide; MAPK - mitogen activated protein kinase; Mek1/2 - dual specificity mitogen-activated protein kinase 1; NF-κB - nuclear factor kappa-B; PC – prostate carcinoma; SAPK/JNK - stress-activated protein kinase/Jun-N-terminal kinase; TNF-α - tumor necrosis factor alpha
ABSTRACT

A key anti-apoptotic transcription factor, NF-κB, is known to be critically important for tumor cell growth, angiogenesis and development of metastatic lesions. We and others showed previously that NF-κB transcription factor was constitutively activated in androgen-independent prostate carcinoma (PC) cell lines due to the up-regulated activity of IκB-kinases (IKK). In this work using Luciferase assay, EMSA and Northern blot analysis of expression of endogenous κB-responsive genes, we demonstrate that a novel highly specific small molecule IKK inhibitor, PS1145, efficiently inhibited both basal and induced NF-κB activity in PC cells. We found that PS1145 induced caspase 3/7-dependent apoptosis in PC cells and significantly sensitized PC cells to apoptosis induced by TNF-α. We also showed that PS1145 inhibited PC cell proliferation. Effects of PS1145 on proliferation and apoptosis correlated with inhibition of IL-6, Cyclin D1, Cyclin D2, IAP1, and IAP2 gene expression and decreased IL-6 protein levels. In addition, we found that incubation with PS1145 inhibited the invasion activity of highly invasive PC3-S cells in invasion chamber assay in a dose-dependent manner. Overall this study provides the framework for development of novel therapeutic approach targeting NF-κB transcription factor to treat advanced PC.
INTRODUCTION

One of the contributing factors to high mortality rate from prostate cancer is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in prostate carcinoma (PC) cells could play a strategic role for PC treatment.

NF-κB transcription factor mediates one of the central signaling pathways protecting cells from apoptotic death (Kucharczak et al., 2003; Karin & Lin, 2002). NF-κB also regulates tumor development through transcriptional regulation of wide variety of genes that encode anti-apoptotic proteins, cell cycle-related proteins, proteins involved in angiogenesis, invasion and metastasis (Karin et al., 2002; Shishodia & Aggarwal, 2004; Ghosh & Karin, 2002).

The active NF-κB complex is a homo- or heterodimer composed of proteins from the NF-κB/Rel family: NF-κB1 (p50/105), NF-κB2 (p52/100), RelA (p65), RelB, and c-Rel (Baldwin, 1996; Verma et al., 1995). In non-stimulated cells, NF-κB resides in the cytoplasm in a complex with the inhibitor protein, collectively called IκB. Several IκB proteins have been identified including IκBα, IκBβ, and IκBε, and proteins p105 and p100, the precursor molecules for NF-κB proteins p50 and p52 respectively (Verma et al., 1995; Whiteside & Israel, 1997). Most agents that activate NF-κB employ a common pathway that involves the phosphorylation of the two N-terminal serines in IκB molecules IκBα, IκBβ, IκBε, and the subsequent ubiquitination and degradation of IκB proteins by the 26S proteasome (Whiteside & Israel, 1997). Signal-induced phosphorylation of IκB is executed by large 900 kD IKK complex, containing two major IκB kinases (IKK) IKKα and IKKβ as well as several scaffolding proteins (Karin & Ben-Neriah, 2000).
There is mounting evidence that constitutive NF-κB activation is a common feature of a variety of hematological and solid tumor cell lines and tumors (Karin et al., 2002; Rayet & Gelinas, 1999), and that constitutive activation of NF-κB suppresses the susceptibility of tumor cells to apoptosis induced by radio- and chemotherapy. We and others showed previously that NF-κB was constitutively activated in androgen-independent PC cell lines due to the constitutive up-regulated activity of IKK kinases (Gasparian et al., 2002; Chen & Sawyers, 2002; Suh et al., 2002; Zerbini et al., 2003). NF-κB inhibition with a IκBα super-repressor in prostate carcinoma (PC) cells led either to apoptosis or to sensitization to apoptosis induced by TNF-α and some other treatments (Herrmann et al., 1997; Muenchen et al., 2000; Gasparian et al., 2002). Most importantly, inhibition of NF-κB with an IκBα superrepressor in PC cells suppressed both growth and development of metastatic lesions by those cells in vivo when they were injected into prostate orthotopically (Huang et al., 2001). Overall these data suggest that NF-κB signaling pathway is critically important for PC cell growth and the development of metastases in animal models. Thus, NF-κB represents an important target for PC treatment, especially when NF-κB inhibition is used in combination with other pro-apoptotic chemotherapeutical drugs.

The results of immunostaining of human PCs for major NF-κB protein RelA/p65 are in line with the data obtained in PC cells in vitro. We and others showed that p65 has nuclear localization in significant number of epithelial cells in prostate tumors especially in hormone refractory metastatic PCs (Gasparian et al., 2002, Sweeney et al., 2004; Shukla et al., 2004; Ross et al., 2004). Nuclear localization of p65 strongly suggests that NF-κB is activated in human PCs, and that constitutive activation of this key anti-apoptotic factor could significantly contribute to the resistance of hormone refractory PCs to apoptosis induction during chemo- and radiotherapy.
There are several pharmacological approaches to target NF-κB. They include repression of NF-κB transactivation potential, stabilization of IκB inhibitors by proteasome inhibitors, and more recently inhibition of upstream IKK kinases (Karin, 2004). The unique properties of IKKβ among other serine-threonine kinases, allowed successful development of specific IKKβ inhibitors at Millennium Pharmaceuticals Inc. (Hideshima et al., 2002; Castro et al., 2003; Lam et al., 2005) and other companies (Burke et al., 2003; Kishore et al., 2003; Ziegelbauer et al., 2005). In the presented work, we studied the effect of the small molecule IKK inhibitor, PS1145 on the status of NF-κB in PC cells, PC cell growth, sensitivity of PC cells to apoptosis, and on invasion capability of PC cells. We demonstrated that PS1145 efficiently inhibited both basal and induced NF-κB activity in PC cells. PS1145 induced apoptosis in PC cells and significantly sensitized PC cell lines to TNF-α induced apoptosis in caspase3/7-dependent manner. In addition, we found that pre-incubation with PS1145 inhibited PC cell growth and the invasion activity of highly invasive PC3-S cells in invasion chamber assay.

RESULTS

Expression of Activated IKKs in Prostate Carcinomas.

Our previous studies as well as data by others revealed that androgen-independent PC cells maintain the high level of NF-κB basal activity due to constitutive IKK activation (Gasparian et al., 2002). To extend our in vitro observations, we performed immunostaining of ten high grade prostate carcinomas (Gleason score 7-9) with Ab against IKKα/β phosphorylated at Ser176/180. It is known that activation of IKKα and IKKβ requires their phosphorylation at those specific serines in the activation loop of IKK kinases (Karin & Ben-Neriah, 2000; Huynh, 2000). As shown in the
Figure 1, activated IKKα/β were strongly expressed in the cytoplasm of epithelial cells in PCs. This finding suggests that IKK kinases are indeed constitutively active in prostate cells in tumors and correlates well with nuclear localization of p65/RelA and NF-κB activation in PCs previously described in our work and in other publications (Gasparian et al., 2002, Sweeney et al., 2004; Shukla et al., 2004; Ross et al., 2004).

**PS-1145 Inhibited Basal and Induced IκBα phosphorylation and NF-κB Activity in Androgen-independent PC cell lines.**

We and others have shown that NF-κB is constitutively activated in androgen-independent PC cell lines due to the constitutive up-regulated activity of IKK kinases. The IκBα protein, a key substrate for IKKα/IKKβ kinase complex, is constitutively phosphorylated in PC3 and DU145 cells (Gasparian et al., 2002). Despite the high constitutive level of NF-κB activity, androgen independent PC cells appeared to be highly sensitive to diverse NF-κB inducers (Gasparian et al., 2003). For example, DU145 cells are highly responsive to TNFα and LPS, while PC3 cells are highly responsive to LPS and TPA but not to TNF-α. Based on these findings, we selected different NF-κB inducers to study the effect of PS1145 on NF-κB activation in PC3 and DU145 cells.

To evaluate the effect of the IKK inhibitor, PS1145 on phosphorylation of IκBα, we performed western blot analysis using whole-cell proteins from DU145 or PC3 cells treated with PS1145 at concentrations 0.5-20 μM for 2-24 hr. We found that PS1145 effect was especially pronounced when cells were incubated with this IKK inhibitor at concentrations 10-20 μM (Fig. 2 A and data not shown). The significant decrease of IκBα constitutive phosphorylation was revealed 12-16 hrs after treatment in both DU145 and PC3 cells (Fig. 2 A-B and data not shown). Furthermore, IκBα
phosphorylation was almost completely blocked when cells were incubated with PS1145 for 24 hrs (Fig. 2 A and B). However, in PC3 cells decrease in IκBα phosphorylation 24 hr after PS1145 treatment coincided with inhibition of total IκBα expression at this time point (see below), suggesting that the relative level of inhibition of IκBα phosphorylation (ratio P-IκBα: total IκBα) in PC3 cells was similar after 16 and after 24 hrs of treatment with PS1145.

PS1145 also strongly inhibited induced IκBα phosphorylation. Indeed, pre-incubation of PC cells with PS1145 for 3 hrs significantly inhibited IκBα phosphorylation induced by short treatments with different compounds such as TNF-α, LPS and TPA (Fig. 3, and data not shown). In addition we found a stable strong effect of long cell pretreatment with PS1145 (24 hr) on TNF-α induced IκBα phosphorylation in DU145 cells (Fig. 2B). Importantly, the similar decrease in IκBα phosphorylation was observed in PC3 cells stably infected with lentivirus expressing IKKβ dominant negative mutant (Fig. 2A, last lane).

We have to mention that treatment of PC3 cells with PS1145 for 16-24 hrs as well as the transfection with exogenous IKKβ d.n. mutant has resulted in the decreased level of total IκBα. This reflects the dramatic down-regulation of IκBα gene transcription in PC3 cells under this treatment (Fig. 5B) and will be discussed later. PS1145 did not affect IκBα expression in DU145 cells (Fig. 2B).

In our next experiments we examined PS1145 effect on NF-κB DNA-binding in PC cells. The effect of PS1145 on different steps of NF-κB activation was dose-dependent, and more pronounced when we used PS1145 at the concentration of 20 μM (data not shown). As shown in the Figure 3, pretreatment of DU145 cells with PS1145 for 3 hrs strongly inhibited IκBα phosphorylation, delayed the degradation of IκBα and nuclear translocation of p65 induced by TNF-α, and accordingly significantly decreased the effect of TNF-α on κB DNA binding.
Similarly, PS1145 inhibited IκBα phosphorylation, degradation, p65 nuclear translocation and NF-κB binding induced by LPS (Fig. 3B) and TPA (data not shown) in PC3 cells.

To evaluate the effect of PS1145 on gene transcription we used transient transfection of PC cells with 5 x κB luciferase-reporter. To induce NF-κB-dependent transcription of reporter gene PC cells were transfected with exogenous IKKβ or treated with the appropriate κB inducer: TNFα (for DU145 cells) or LPS (for PC3 cells) for 24 hrs. NF-κB activity induced by TNF-α, LPS and exogenous IKKβ was strongly inhibited by PS1145 in both PC cell lines (Fig. 4A and B). In addition, PS1145 significantly blocked basal NF-κB transcriptional activity in PC3 cells (Fig. 4B).

To study the effect of PS1145 on the transcription of endogenous κB-responsive genes we used Northern blot analysis of IκBα expression. IκBα gene has five κB sites in its promoter, and is tightly regulated by NF-κB in different cells (Ito et al., 1994). We showed previously that the level of steady-state IκBα expression directly correlated with the level of constitutive NF-κB activity in different PC cell lines (Gasparian et al., 2002). The results of Northern blotting demonstrated that treatment with PS1145 (20 μM) for 24 hrs blocked both basal and inducible expression of IκBα in PC cells (Fig. 5A and B). The effect of PS1145 on IκBα expression was dose- and time-dependent (data not shown) with maximum IκBα expression blockage after 24 hrs exposure to PS1145.

Overall the results of Northern blot analysis correlated well with the data obtained by Luciferase reporter assay and EMSA. We found that PS1145 strongly inhibited NF-κB activity in both PC cell lines irrespectively of the nature of the NF-κB inducer. Notably, the effect of PS1145 on basal NF-κB activity was revealed easier in PC3 cells despite the fact that PS1145 strongly inhibited basal IκBα phosphorylation in both PC cell lines.
PS1145 Inhibited Proliferation of DU145 cells.

The effect of PS1145 on proliferation was assessed by several approaches in DU145 cells. As shown in the Figure 6A, MTT test revealed 30-35% decrease in DU145 cell numbers 48-72 hrs after PS1145 treatment. BrdU labeling of DU145 cells confirmed the result of MTT test. The number of BrdU-positive cells (cells in S-phase) was decreased by 32±3.25 % in DU145 cell cultures treated with PS1145 for 72 hrs (Fig. 6B). The inhibition of proliferation was further confirmed by the decrease of Ki67 protein expression known to be present in cells in G1, S, G2 and M phases but not in G0 phase of the cell cycle (data not shown). Therefore we have shown that PS1145 caused significant inhibition of proliferation in DU145 cells.

PS1145 Induces Apoptosis in DU145 cells and Increases their Sensitivity to TNFα.

Long-term exposure to PS1145 was toxic for DU145 cells. To evaluate the effect of PS1145 on apoptosis in these cells we measured caspase3/7 activity, and used Western blot analysis to assess poly-(ADP-ribose) polypeptide (PARP) cleavage. As shown in the Figure 7A, treatment of DU145 cells with PS1145 for 48 hrs resulted in strong activation of caspase 3/7 in a dose dependent manner. Western Blot analysis of caspsase 3/7-dependent PARP cleavage also demonstrated that PS1145 induced apoptosis in DU145 cells 48-72 hr after the beginning of the treatment (Fig 7B, C). Importantly, PS1145 sensitized DU145 cells to TNFα-induced apoptosis: PARP cleavage and caspase 3/7 activation (Fig. 7B, C) were much more pronounced in DU145 cells treated with combination of TNF-α and PS1145. These data are in line with the previous observations that NF-κB protects different cells, including PC cells, against apoptosis induced by TNF-α, and that NF-κB blockage by different genetic approaches results in cell sensitization to TNF-α (Muenchen, 2000, Gasparian et al., 2002, Orlowski et al., 2002; Shukla & Gupta, 2004).
Overall our data indicate that the effect of PS1145 on PC cell growth and apoptosis develops after prolonged treatment (i.e. requires cell maintenance under the conditions when NF-κB is chronically inhibited), even though the significant NF-κB inhibition is achieved in 16 hrs.

**PS1145 Inhibited the Invasion Activity of PC3-S cells *in vitro*.**

It is known that DU145 cells do not possess high invasion and migration capability *in vitro* and *in vivo* experiments. In contrast, several clones derived from the original PC3 cell line were reported to be highly invasive in animals and in invasion chambers *in vitro*. Thus, to study the effect of PS1145 on PC cells motility and invasiveness, we used a highly invasive PC3 clone PC3-S (Lindholm *et al.*, 2000). The cell invasiveness was studied using invasion assay of radioactively labeled cells. As shown in the Figure 8, PS1145 dramatically inhibited invasion of PC3-S cells in a dose-dependent manner. The effect was more pronounced if the cells were pre-incubated with PS1145 for 24 hrs. The inhibition of invasion by PS1145 was not due to PS1145 toxicity for PC3-S cells. PC3-S cells appeared to be rather resistant to the toxic effect of PS1145 evaluated by flow-cytometric analysis with propidium iodide (data not shown).

**PS1145 Effects on Gene Expression in PC cells.**

NF-κB regulates wide variety of genes that encode anti-apoptotic proteins, cell cycle proteins, cytokines, proteins involved in cell-cell and cell- extracellular matrix (ECM) interactions and others. Thus, to address the mechanisms underlying the effects of PS1145 on PC cells, we performed semi-quantitative RT-PCR analysis of the set of 21 genes known to be important for control of cell cycle, apoptosis and cell-cell and cell- ECM interactions (Martone *et al.*, 2003; Toth, 1995; Catz, 2001; Glasgow *et al.*, 2000; Hinz, 2001; Gupta *et al.*, 2002). The gene selection was made after the comparison of database obtained by cDNA array analysis of global effect of
PS1145 on gene expression in PC cells (these data are not shown in this paper, and are planned to be used for another research project) and the literature database for NF-κB-dependent genes (http://www.nf-kb.org). The list of the selected genes is presented in the Table 1. The quantitative analysis of gene expression was performed by Agilent 2001 Bioanalyzer as described in Materials and Methods.

As shown in the Figures 9 and 10 we found that the expression of nine out of 21 studied genes was significantly changed in DU145 cells treated with PS1145, especially after longer 72 hr treatment. Figure 9 represents the agarose electrophoresis analysis of RT-PCR products. Figure 10 shows quantitative analysis of expression of all the selected genes (Table 1). As expected, the expression of well-known NF-κB-dependent genes such as inhibitor of apoptosis IAP-1, IAP-2, cyclinD1, cyclinD2, IL-6, and IL-9 was significantly decreased in DU145 cells with inhibited NF-κB activity. The similar inhibition of those genes was found in PC3 cells (data not shown). The expression of three other apoptosis-related genes c-FLIP (CASP8 and FADD-like apoptosis regulator precursor, long isoform), XAF1 - XIAP (X-linked inhibitor of apoptosis protein)-associated factor, and cell death regulator AVEN was increased in both PC cell cultures, while we did not find any changes in expression of Bcl-2 and Bax. The relevance of those changes to apoptosis induced by PS1145 in PC cells will be discussed below. We also did not find significant changes in the expression of cyclinB1 and B2, and Cdk5 in PC cells, even though some of those cell cycle-related genes have been previously reported to be down-regulated by NF-κB inhibitors (Guttridge et al., 1999; Gupta et al., 2002). Genes from VEGF (vascular endothelial growth factor) family were differentially regulated in two PC cell lines: VEGF-C was significantly down-regulated only in DU145 cells and VEGF-D was down-regulated only in PC3 cells (Fig. 10). Overall, anti-apoptotic and anti-proliferative effect of PS1145 correlated well with down-regulation of IAP-1, IAP-2, cyclinD1 and D2.
It is known that cytokine IL-6 plays an important role in the growth of androgen independent prostate tumor cells via autocrine and paracrine mechanisms (Zerbini et al., 2003; Giri et al., 2001; Culig et al., 2004). To extend our finding on the inhibition of IL-6 gene expression by PS1145 in DU145 cells, we used ELISA assay to evaluate the amount of IL-6 protein secreted into the cell culture medium by DU145 cells treated with PS1145. As shown in the Fig. 11, inhibition of IKK resulted in a significant decrease of IL-6 levels.

Effect of PS1145 treatment on cell signaling pathways

The data obtained in our studies indicate that despite the strong effect of PS1145 on NF-kB activity, its effect on proliferation and apoptosis in PC cells was more modest. This raised the question about possible activation of some pro-proliferative such as MAPK (Zerbini et al., 2003) and anti-apoptotic such as Akt (Culig et al., 2004; Li et al., 2005) signaling pathways in PC cells to compensate for NF-kB blockage. To assess the effect of PS1145 on Akt, MAPK and SAPK/JNK signaling we evaluated the level of Akt, SAPK/JNK and Mek1/2 phosphorylation in DU145 cells treated with PS1145 for 24 and 72 hrs. As shown in the Figure 11, there was no change in phosphorylation level of SAPK/JNK kinases. Against expectations, Akt activity was inhibited by long, 72 hr treatment of DU145 cells with PS1145. Interestingly, phosphorylation of c-Raf and down-stream Mek1/2 was strongly, even though temporarily increased in response to PS1145 treatment. This correlated very well with increased phosphorylation of down-stream Mek1/2 target kinases Erk1/2, that was mostly pronounced 24 hrs after treatment with PS1145.

DISCUSSION
There is mounting evidence that NF-κB activation is associated with tumorigenesis. NF-κB was found to be activated in human leukemias and lymphomas, lung and breast carcinomas as well as in numerous cell lines of different origin (Karin et al., 2002; Rayet & Gelasas, 1999). The chronic activation of NF-κB in tumor cells has been linked both to genetic changes and to epigenetic mechanisms. There are numerous reports indicating that upstream signaling pathways causing (or associated with) tumor development can activate NF-κB. Viral oncoproteins including Tax and EVB nuclear antigen are known to activate NF-κB through interaction with IKK complex or some other mechanisms (Orlowski & Baldwin, 2002, Karin et al., 2002). NF-κB and IKK complex could be induced by activated oncogenes Ras, Bcr-Abl, members of Rho protein family (Orlowski & Baldwin, 2002). Production of numerous growth factors and cytokines that are strong activators of IKK complex, and whose expression is in turn NF-κB-dependent, is typical for tumor cells (Orlowski & Baldwin, 2002; Greten & Karin, 2004; Zerbini et al., 2003). Those cytokines including IL-6 and growth factors may contribute to the establishment of positive autocrine/paracrine loops of NF-κB activation in tumor cells (Zerbini et al., 2003; Giri et al. 2001; Culig et al., 2004). There is also evidence that IKK-independent pathways, including p65 phosphorylation can be involved in NF-κB constitutive activation in tumor cells (Viatour et al., 2005).

Recently we and others found that NF-κB is activated in androgen-independent PC cells and in prostate tumors where NF-κB has nuclear localization in at least 15% of cells (Palayoor, 1999, Gasparian et al., 2002, Sweeney et al., 2004; Shukla et al., 2004; Ross et al., 2004). The major mechanism of NF-κB activation in PC cell lines involves the aberrant activation of IKK complex resulting in increased phosphorylation and instability of IκB inhibitor proteins (Gasparian et al., 2002). Importantly, in this work using immunostaining with antibodies against activated, phosphorylated IKKs we showed for the first time that IKK complex is also activated in prostate
carcinoma samples. Thus, IKKs, especially IKKβ that is critical for NF-κB activation, represent a novel important target for NF-κB blockage in PC and other tumor cells. Very recently, several pharmaceutical companies have started working on the design of potent orally active IKKβ inhibitors (Burke et al., 2003; Kishore et al., 2003; Ziegelbauer et al., 2005; Baxter et al., 2004; Murata et al., 2004). PS1145 is one of these highly specific IKK inhibitors (IC < 0.1 μM) recently developed by Millenium Pharmaceuticals, Inc. (Hidestima et al., 2002; Lam et al., 2005).

In this work we developed a comprehensive picture of the effects of PS1145 on NF-κB activity, growth, sensitivity to apoptosis and invasiveness of PC cells. We first demonstrated that pretreatment with PS1145 (10-20 μM) efficiently inhibited both basal and induced NF-κB activity in PC cells. Then we studied the major biological responses of PC cells resulting from NF-κB inhibition. We showed that PS1145 inhibited proliferation of DU145 cells when cells were incubated with IKK inhibitor for 48-72 hrs. These data are in line with the previous findings indicating that NF-κB is an important regulator of cell proliferation, and its effect is mediated through regulation of expression of cyclins (especially cyclinD1), possibly CDK/CKI genes, and some other cell cycle-related genes for example, c-myc. Our study has revealed that PS1145 significantly inhibited cyclinD1 and cyclinD2 expression in PC cells, but did not affect the expression of cyclin B1, cyclin B2, cdk6, cdk4, cdc2, cdc5, and cdc6. Relatively modest inhibition of PC cell proliferation by PS1145 correlates well with the results obtained in multiple myeloma cells treated with the same IKK inhibitor (Hidestima et al., 2002), and in PC-3 cells transfected with IκBα superrepressor (Huang et al., 2001) and may reflect the existence of compensatory mechanisms that counteract NF-κB blockage in PC cells. Indeed, we found that Raf/Mek1/2/Erk1/2 kinases were strongly activated in DU145 cells by PS1145. This suggests that simultaneous inhibition of NF-κB and MAP kinase cascade may result in more profound inhibition of PC cell proliferation.
A key role of NF-κB in cell protection against diverse apoptotic stimuli including chemotherapeutic and radiotherapy is very well known. The anti-apoptotic NF-κB-regulated genes include genes that encode Bcl-2-like proteins (Bfl1, Bcl-XL, and Nr13), inhibitor of apoptosis proteins - IAP-1, IAP-2, X-IAP1, and others (Barkett & Gilmore, 1999). In our experiments PS1145 itself triggered modest apoptosis in DU145 cells treated for 72 hrs or longer. Most importantly, PS1145 treatment significantly sensitized relatively resistant DU145 cells to TNFα-induced apoptosis. These data correlate well with the previous findings that NF-κB blockage by overexpression of non-degradable IκBα mutant may result in apoptosis or in sensitization to TNF-α-induced apoptosis in PC cells. Further analysis of mechanisms of apoptotic death induced by PS1145 in PC cells revealed the central role of caspases 3/7 in this process. Indeed, we found that PS1145 induced caspase 3/7 activation, and consequently, increased cleavage of PARP, a target protein for caspase 3/7. In turn, the activation of caspase 3/7 correlated with the decreased expression of its inhibitors, IAP-1 and IAP-2 after PS1145 treatment. Moreover, our data indicated the role of XIAP-associated factor-1 (XAF1), an antagonist of another inhibitor of caspase3/7, x-IAP. We found that XAF1 expression was significantly increased in PC cells treated with PS1145. On the contrary, we did not find changes in the expression of genes involved in mitochondrial apoptosis (Rapp et al., 2004; Abraham & Shaham, S, 2004). Unexpectedly, certain anti-apoptotic genes have been activated in PC cells after PS1145 treatment. For example, the expression of genes that encode caspase-8 (FLICE) inhibitory protein c-FLIP (long FLIP isoform), and especially cell death regulator Aven was increased in DU145 cells treated with PS1145. Aven was recently shown to bind both Bcl-x(L) and the caspase9 regulator Apaf-1, thus inhibiting mitochondrial apoptosis (Chau et al., 2000; Peter, 2004, Figueroa et al., 2004). Interestingly, the effect of PS1145 on Aven expression was especially pronounced in PC3 cells more resistant to PS1145-induced apoptosis, than in DU145 cells (data not
shown). This may potentially explain known higher resistance of PC3 cells to apoptosis induced by NF-κB blockage.

As we mentioned, the specific pharmacological IKK inhibitors have been developed only recently. Thus, the information about their effect on tumor cell behavior is very limited. PS1145 was recently tested in multiple myeloma cells (Hideshima et al., 2002). Another novel IKK inhibitor, BMS-345541 (Burke et al., 2003) was studied in human melanoma cells (Yasui et al., 2003). Overall, the effects of IKK inhibitors in other tumor cells were similar to our findings in PC cells. Both IKK inhibitors decreased tumor cell proliferation in vitro, and either induced apoptosis or sensitized tumor cells to apoptosis induced by TNF-α. BMS-345541 also inhibited melanoma cell growth in vivo as xenografts in nude mice. Interestingly, in both cell types IKK inhibition has resulted in abrogation of paracrine growth loops, mediated in multiple myeloma cells by IL-6, and in melanoma cells by chemokine CXCL1. Aberrant expression of IL-6 has been implicated in PC progression and resistance to chemotherapy (Zerbini et al., 2003; Giri et al., 2001; Culig et al., 2004). IL-6 is highly expressed in androgen-independent PC cell lines, and has been shown to function as an important growth factor in PC cells possibly also through autocrine growth loop (Zerbini et al., 2003). Importantly, treatment of PC cells with PS1145 resulted in significant decrease of IL-6 gene expression and decreased concentration of IL-6 protein in cell culture medium, suggesting that PS1145 affects positive growth loop mediated by IL-6 in PC cells.

In conclusion, the presented results obtained in PC cell cultures suggest that constitutively active anti-apoptotic and pro-proliferative NF-κB signaling represents a rational target for PC treatment, especially in combination with some other pro-apoptotic chemotherapeutical drugs. The development of IKK inhibitors that more specifically block NF-κB signaling than all other agents including proteasome inhibitors, will be very helpful to block NF-κB as a novel anti-cancer strategy in clinics.
MATERIALS AND METHODS

Cell Cultures and Treatments.

DU145 and PC3 cells were purchased from American Tissue Culture Collection (Rockville, MD). DU145 and PC3 cells were cultured in RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), sodium pyruvate (1mM, Sigma Chemical Co., St. Louis, MO), and antibiotics (Gibco BRL Life Technologies, Rockville, MD). The following reagents were used for cell treatments: PS1145 (Millenium Pharmaceuticals, Inc, Boston MA), LPS, DMSO and TNF-α from R&D Systems (Minneapolis, MN). PS1145 was dissolved in DMSO, and stock solution was stored at −20° C. PC3 cells stably expressing d.n. IKKβ mutant tagged with FLAG were generated using lentivirus system from Invitrogen Corporation (Carlsbad, CA) following the manufacture’s protocol. Antibiotic blasticidin was used at concentration of 6 μg/μl to select for d.n. IKKβ expressing clones. The d.n. IKKβ expression was confirmed using anti-FLAG antibodies (Sigma Chemical Co., St. Louis, MO) and anti-IKKα/β antibodies (Santa Cruz Biotechnology Santa Cruz, CA) by western blotting and immunostaining (data not shown). We used in our work pulled PC3-d.n. IKKβ cell cultures.

Preparation of cellular extracts and electrophoretic mobility shift assay (EMSA).

Nuclear and cytosolic proteins were isolated as described previously (Lyakh et al., 2000). The binding reaction for EMSA contained 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1mM EGTA, 6% glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of sonicated salmon sperm DNA, γ-32P-labeled (2 – 3 × 10⁵ cpm) double-stranded κB-consensus oligonucleotide (Promega Corp., Madison, WI), γ-32P-labeled (2 – 3 × 10⁵ cpm) and 5-10 μg of the nuclear extract. DNA-binding reaction was
performed at room temperature for 30 minutes in a final volume of 20μl. DNA-protein complexes were analyzed on 6% DNA retardation gels (Novex, Carlsbad, CA). Dried gels were subjected to radiography.

Western blot analysis.

Whole cell protein extracts were prepared using RIPA buffer as described elsewhere (Rosenberg, 1996). Proteins were resolved by electrophoresis on 10% SDS-PAGEs and transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA). Anti-phospho-Mek1/2, anti-phospho-Erk1/2, anti-phospho-Akt, anti-phospho-SAPK/JNK, anti-phospho-c-Raf and anti-phospho-Ser32 IxBα and anti-PARP Abs were from Cell Signaling Technology, Inc (Beverly, MA); anti-p65 and anti-IxBα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were blocked with 5% non-fat milk in TBST buffer and incubated with primary antibodies overnight at 4 °C. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) were used as secondary antibodies. ECL Western blotting reagent (Amersham Pharmacia Biotech, Sweden) was used for protein detection. To verify for equal amounts of proteins loaded and transferred the membranes were stained with Ponceau Red.

Transfection of PC cells and Luciferase activity.

The following constructs were used for transfections: κB-luciferase reporter – Firefly luciferase (FL) under 5 x κB promoter kindly provided by Dr. W.C. Greene (Gladstone Institute for Virology and Immunology, University of California, San Francisco, CA); pRL-null construct – Renilla luciferase (RL) under minimal promoter (Promega Corp., San Luis Obispo, CA); pcDNA3.1-CMV-IKKβ w.t. (kindly provided by Dr. F. Mercurio, Signal Pharmaceutical, Inc., San Diego, CA). PC cells were plated onto 12 well plates and at 50% confluency were co-transfected
with indicated plasmids using Effectene reagent (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The amount of each plasmid DNA was 0.5 μg/well. Prostate cells were harvested 36 hrs after the transfection and the Luciferase activity was measured by dual luciferase assay (Promega Corp., San Luis Obispo, CA) on a TD20/20 Turner luminometer (Turner Design Instruments, Sunnyvale, CA). When necessary, before transfections cells were pre-treated with PS1145 (10 μM) and/or TNFα (7 ng/ml) or LPS (1.5 μg/ml). FL activity was normalized against RL activity to equalize for transfection efficacy.

**Northern blot analysis.**

Total RNA from freshly harvested cells was isolated by TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and subjected to Northern blotting. Twenty μg of total RNA was resolved in 1% agarose - 6% formaldehyde gel. The RNA was transferred to nylon membranes and probed for IκBα. The DNA probe was prepared by random-primed reactions using the complete coding sequence of human IκBα cDNA (ATCC, Rockville, MD).

**RT-PCR.**

Two step RT-PCR reaction using reverse transcriptase MLV-RT with random primers and PCR-Supermix (both from Invitrogen Corp., Carlsbad, CA) with appropriate PCR primers was performed using total RNA isolated by RNA easy mini kit (Qiagen Inc., Valencia, CA). PCR primers were designed using Primer-Bank database (http://pga.mgh.harvard.edu/primerbank/) and RTPrimerDB Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/index.php). PCR products were analyzed by electrophoresis in 1.5% agarose gel. The actual amount of PCR product was measured by an Agilent 2001 Bioalyzer and normalized to the amount of GAPD PCR product. For quantitative analysis the data are represented as the ratio of
GAPD-normalized amount of PCR product in PS1145-treated cells to the GAPD-normalized amount of PCR product in control cells.

**IL-6 secretion.**

DU145 cells were plated at 5,000 cells/well in BD356640 Poly-D Lysine 96 well plates (Beckton Dickenson, Franklin Lakes, NJ). Cells were incubated with PS1145 at increasing concentrations for 48 hr. IL-6 level in tissue culture media was measured using Quantikine Assay for human IL-6 (R&D Systems, Minneapolis, MN) according to manufacturer’s protocol using Wallace Victor 2 1420 luminometer.

**Assessment of Proliferation.**

Cell proliferation was assessed using None-Radioactive Cell Proliferation Assay (MTS test) from Promega Corporation (San Luis Obispo, CA) and bromodeoxyuridine (BrdU) cell labeling using immunofluorescence. For both tests PC were plated onto 12 well plates (20x10^3 cells/well), and cultured for 24-72 hrs in the presence of 10 μM PS1145. Every 24 hrs fresh complete media containing 10 μM PS1145 was added to the cells. For MTS test each group of cells was plated in triplicate. The MTS reagent was prepared and used according to the manufacture’s protocol. Optical density of the samples was measured on a plate reader at 490 nm.

For BrdU labeling the cells were plated onto sterilized cover slips placed on the well bottoms in 12 well plates. PS1145-pretreated and control DU145 cell cultures were treated with BrdU (2μg/ml) for 2 hours. The cover slips with cells were fixed and permeabilized with acetone-methanol (1:1) mixture at -20° for 15 minutes, washed with PBS, blocked with 20% goat serum and stained with primary anti-BrdU Ab from Becton Dickinson (Franklin Lakes, NJ) and secondary goat anti-mouse Ab conjugated with Cy3 (Jackson Immunoresearch laboratories Inc.,
West Grove, PA). DAPI (Vector laboratories, Burlingame, CA) was used to counterstain the nuclei. The number of BrdU positive cells was counted in PS1145 treated and control cultures (ten fields of view in each sample). For quantitative analysis the data were presented as the % of BrdU-positive nuclei to the total number of nuclei stained with DAPI.

**Apoptosis detection.**

To evaluate apoptosis we used Western blot analysis of PARP proteolysis and Caspase3/7 functional assay. To study PARP proteolysis, prostate cells, plated on 10 mm dishes, were treated with 10 μM PS1145 alone or in combination with 7.5 ng/ml TNF-α (R&D Systems, Minneapolis, MN) for 16-72 hrs upon reaching 50% confluence. Adherent cells and detached floaters were combined for whole-cell protein extract preparations. PARP cleavage was estimated by Western blot analysis with anti-PARP antibody (PharMingen, San Diego, CA).

For caspase 3/7 functional assay, DU145 cells were plated at 5 x 10^3 cells/well of a 24 well plate in 100 μl of complete media. PS1145 was added at increasing concentrations from 0.1 to 50 μM. Caspase activity was measured after 48 hr treatment with PS1145 using ApoOne kit (Promega Corp., Madison, WI) according to the manufacture’s protocol. 100 μl of the substrate were added for 2.5 hrs.

**Transwell® Invasion Assays.**

The PC-3 High Invasion subclone (PC3-S) was previously selected by serial passages through reconstituted basement membrane Matrigel® (Becton Dickinson, Lincoln Park, NJ), in the Transwell® invasion apparatus (Lindholm et al., 2001). For analysis of PC-3S cell invasion in the presence of PS1145 inhibitor, a Transwell® invasion assay was used according to the protocol described previously (Lindholm et al., 2001). Prior to the invasion assay, the cells were pre-
incubated with \[^{3}H\]-thymidine and either PS1145 or control vehicle (Lindholm et al., 2000) overnight. The cells were incubated in the invasion chamber for up to 72 hours. Invading cells were collected as described previously (32), and the cell invasion was quantitated by counting the cell-associated cpm. The percent invasion was calculated by dividing the invading cell-associated cpm to the total cell-associated cpm. The statistical analysis of percent invasion was determined by Student’s t-test comparisons using InStat™ statistical software (GraphPad Software, Inc., San Diego, CA). The results are presented as mean ± standard deviation (SD).

**Immunostaining of prostate tissues.**

Prostate tissues were obtained from white male patients at the age 40-82 years during biopsy or surgery to remove prostate tumors. Paraffin sections of formalin-fixed prostate carcinoma samples with verified diagnosis were used for immunostaining. The immunostaining was performed using Envision+ System-HRP (DAB) kit according to the manufacture’s protocol (DakoCytomation, Carpenteria, CA). After Ag retrieval in a pressure cooker (for 5 minutes at 20-25 psi) in citric buffer (pH 6.0), tissue sections were blocked with 20% goat serum in PBS, and consequently incubated with primary rabbit polyclonal Ab against phospho-IKKα/β (Cell Signaling Technology, Inc., Beverly, MA) followed by secondary anti-rabbit IgG-reagent provided with the Envision+ System-HRP (DAB) kit. Immunostaining was visualized with DAB chromogen (DakoCytomation, Carpenteria, CA) and counterstained in Mayer’s hematoxylin.

Data in all figures are shown as results of the representative experiments. All experiments were repeated at least three times.

**ACKNOWLEDGEMENTS**
We thank Dr. W.C. Greene (Gladstone Institute for Virology and Immunology, University of California, San Francisco, CA) and Dr. F. Mercurio (Signal Pharmaceutical, Inc., San Diego, CA) for their kind gift of plasmids.

Work was supported by DOD prostate grants DAMD17-01-1-0015 and DAMD17-03-1-0522 (to IB), Northwestern University Prostate SPORE Developmental Project (to IB), and Russian Foundation for Basic Research, grant # 0404-49240a (to AG).

Table 1. Genes studied in DU145 and PC3 cells treated with PS1145.

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<th>Gene group</th>
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<td>1. Apoptosis-related genes</td>
<td>IAP1; IAP2; XAF1; BCL2; BAX; AVEN; c-FLIP, AVEN</td>
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<td>2. Proliferation-related genes</td>
<td>Cyclin D1, Cyclin D2, Cyclin B1, Cyclin B2, Cdk4, Cdk6, Cdc2, Cdc5, Cdc6, Cdc25B</td>
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<tr>
<td>3. Angiogenesis-related genes</td>
<td>VEGF-A, VEGF-C, VEGF-D</td>
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<tr>
<td>4. Adhesion molecules</td>
<td>N-cadherin1, ICAM-1</td>
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<tr>
<td>5. Cytokines</td>
<td>IL-6, IL-9</td>
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LEGENDS TO THE FIGURES

Figure 1. IKKα/β are phosphorylated in prostate carcinomas

Immunostaining of prostate carcinoma (Gleason score 9) with antibodies against IKKα/β phosphorylated at Ser176/180 in IKK activation loop. Note the strong expression of phosphorylated IKKs in epithelial cells of PC.

Figure 2. PS1145 inhibited basal and induced IκBα phosphorylation

A. PC3 cells were treated with PS1145 at indicated concentrations (0.5-20 μM) for 2-24 hr. Far right lanes: PC3 cells stably infected with empty lentivirus or lentivirus expressing d.n. IKKβ mutant.

B. DU145 cells were treated with 10 μM PS1145 for 2-24 hrs. Far right lanes: DU145 cells were pretreated with PS1145 for 24 hrs and treated with TNF-α (7 ng/ml) for 1 hr or treated with both agents for 24 hrs.

Western blots containing whole cell protein extracts (50 μg/lane) were probed with anti-IκBα and anti-phospho-IκBα antibodies or anti-actin Ab as a control.

Figure 3. PS1145 inhibited NF-κB binding in PC cells

A. Control and PS1145-pretreated (10 μM x 3 hr) DU145 cells were treated with TNF-α (7 ng/ml) for 10 min. A.1. Nuclear protein extracts were used to evaluate κB binding by electrophoretic mobility shift assay (EMSA); A.2. Western blots containing nuclear protein extracts (20 μg/lane) were probed for expression of p65; A.3-A.4. Western blots containing cytoplasmic protein extracts (20 μg/lane) were probed for expression of IκBα and IκBα-P.
B. Control and PS1145-pretreated (10 μM x 3 hr) PC3 cells were treated with LPS (1.5 μg/ml) for 1 hr. Procedures used in B.1-B.4 are identical to those described in A.1-A.4.

**Figure 4. Effect of PS1145 on NF-κB transcriptional activity in PC cell lines**

DU145 (A) and PC3 (B) cells were co-transfected with 5 x κB Firefly luciferase-reporter (FL), pRNL-null (Renilla Luciferase, RL), pcDNA and with wild type IKKβ plasmids. Cells were treated with TNF-α (7 ng/ml), LPS (10 μg/ml), PS1145 (10 μM), or combination of PS1145 + TNF-α or PS1145 + LPS for 24 hours. Luciferase activity was measured by dual luciferase assay. Data are shown as fold of inhibition or induction, calculated as FL/RL ratio for treated samples normalized against FL/RL ratio for control samples (controls are presented in far left lanes in Figs.4A and 4B)

**Figure 5. Northern blot analysis of IκBα expression in PC cells after PS1145 treatment**

DU1145 (A) and PC3 cells (B) were treated with TNF-α (7 ng/ml), PS1145 (10 μM), or combination of PS1145 + TNF-α for 24 hrs. Northern blots (20 μg RNA/lane) were probed for expression of IκBα. Propidium iodine gel staining (lower panels) was used for the verification of equal RNA loading.

**Figure 6. PS1145 inhibited proliferation of DU145 cells**

A. DU145 cells were treated with 10 μM PS1145 for indicated periods of time, and cell growth was evaluated by MTS test. B and C. Analysis of BrdU incorporation in untreated (B) and treated with 10 mM PS1145 (C) DU1145 cells. Cells were treated with BrdU for 1 hr, fixed in 4%
formaldehyde and used for immunofluorescence with anti-BrdU antibody. DAPI nuclear staining and BrdU staining were evaluated by fluorescent microscope. X 320.

**Figure 7. PS1145 induced apoptosis in DU145 cells**

A. Caspase 3/7 activity was evaluated in DU145 cells by ApoOne kit after cells were treated with increasing concentrations of PS1145 for 48 hr. B. Western Blot analysis of PARP cleavage in DU1145 cells treated with TNF-α (7 ng/ml), PS1145 (10 μM), or combination of PS1145 + TNF-α for 16 and 72 hrs. C. Densitometric profile of Western blot analysis of PARP cleavage. The data presented as a ratio of total cleaved PARP products of 89 kDa and 24 kDa to uncleaved PARP.

**Figure 8. PS1145 inhibited invasion capacity of PC3-S highly invasive clone**

PC3-S cells were pre-treated with 5 and 10 μM PS1145 for 24 hrs and incubated in the Transwell® invasion chamber for up to 72 hours. The data are presented as percent of invasion calculated by dividing the invading cell-associated cpm to the total cell-associated cpm (see Materials and Methods).

**Figure 9. PS1145 effects on the expression of selected genes in DU145 cells**

Agarose gel analysis of RT-PCR products of selected genes in DU145 cells treated with 10 μM PS1145 for 24 or 72 hrs.

**Figure 10. Quantitative analysis of PS1145 effect on gene transcription in DU145 and PC3 cells**

RT-PCR products were quantitatively analyzed using Agilent 2002 Bioanalyzer. The data are shown as GAPDH-normalized fold change factor calculated for each gene as GAPD-normalized
amount of RT-PCR product from PS1145-treated DU145 cells divided by that of untreated DU145 sample.

A. Analysis of expression of cell cycle-related genes in DU145 cells; B. Analysis of apoptosis related genes in DU145 cells. C. Expression of angiogenesis-related genes in PC3 cells. D. Expression of angiogenesis-related genes in DU145 cells.

**Figure 11. Analysis of PS1145 effect on IL-6 protein expression in DU145 cells**

Cell culture medium was harvested 48 hr after treatment of DU145 cells with PS1145 at increasing concentrations, and IL-6 expression was evaluated in cell culture medium by ELISA (Quantikine assay).

**Figure 12. Analysis of PS1145 effect on cell signaling pathways.**

Western blot analysis of whole cell protein extracts (50 μg/well) prepared from DU145 cells treated with 10 μM PS1145 for indicated periods of time. Western blot membranes were probed for expression of phosphorylated c-Raf, Mek1/2, Erk1/2, SAPK/JNK, Akt, and actin as a control for loading.

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**A.**

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Yemelyanov et al. Fig 2 (A, B)
A
-  +  +  TNFα
-  -  +  PS1145
3h pre-treatment
1
NF-κB

2
p65

3
p-IκBα

4
IκBα

B
-  +  +  LPS
-  -  +  PS1145
3h pre-treatment
1
NF-κB

2
p65

3
p-IκBα

4
IκBα

Yemelyanov et al, Fig. 3 (AB)
PS1145  -  +  -  +  -  +  +  -  -  +  +  PS1145
TNFα    -  -  +  +  -  +  +  -  -  +  +  TNFα

A

B

Yemelyanov et al. Fig 5 (AB)
A

Fold change

O.D. (treated sample) / O.D. (control)

24 h  36 h  48 h  72 h

0.0  0.2  0.4  0.6  0.8  1.0

B

C.1 DAPI

DU1145 untreated

C.2 BrdU

C

D.1 DAPI

DU1145 treated with PS1145

D.2 BrdU
DMSO

PS1145

24 h 72 h

GAPD

IAP-1

IAP-2

XAF1

AVEN

c-FLIP

IL-9

IL-6

N-Cadherin1

ICAM-1

Cyclin D1

Cyclin D2

Yemelyanov et al. Fig. 9

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For almost 20 years viral vectors have been widely used for the delivery of foreign genes into target cells. The viral vectors used for gene transfer include adenoviruses, adeno-associated viruses, herpes simplex virus and retroviruses. Each specific vector group has its own advantages and limitations with respect to gene delivery. For example, the use of adenoviruses and herpes simplex virus-based vectors is hampered by their inability to stably integrate into the host genome. In contrast, the retroviral vectors have become increasingly popular because of their ability to integrate into the host cell genome and to maintain persistent gene expression. The retroviral family includes both oncogenic retroviruses and lentviruses. The major drawback of the commonly used oncoviral vectors is their inability to transduce non-dividing cells. It is thought that retroviruses are not able to translocate to the nucleus of non-dividing cells and that integration into the genome only occurs during mitosis. On the
contrary, lentiviruses have the ability to integrate into the cell genome during both mitotic and post-mitotic stages of the cell cycle. Thus, one of the major advantages of lentiviruses is the ability to target non-dividing cells and tissues.

There are several lentiviral genetic systems that have been recently developed and optimized for gene delivery into mammalian cells. Most of these current lentiviral systems use HIV-1-based vectors, some are derived from HIV-2, and others are derived from Feline Immunodeficiency Virus (so-called Felix-vector system) and other viruses. For safety reasons, the production of lentivirus derived vectors involves a genetically split gene expression design that helps to eliminate, or strongly decrease, the risk of the recombination leading to the production of replication-competent virus. Under this design, the viral elements that are specifically required for cell infection and lentiviral production are expressed separately. The viral elements are (i) the lentiviral packaging genes that encode helper proteins including gag-pol genes, (ii) the lentiviral transfer vector RNA containing the transgene expression cassette, and (iii) the heterologous glycoprotein of the viral envelope. The segregated viral genes are maintained in packaging plasmids in bacteria, and the combination of these plasmids is used for transfection of mammalian cells (packaging cells) to produce replication-defective virus stocks.

During the last 3-5 years lentiviral vectors have been successfully used by different laboratories to transduce transformed cell lines, as well as primary cell cultures derived from different tissues. Different cell types including lymphocytes, neurons, hepatocytes, keratinocytes and stem cells from different tissues have been efficiently infected by lentivirus. *in vitro.*

There is an increasing number of reports on successful lentiviral gene delivery *in vivo* to such tissues and organs as cornea, retina and pigment epithelium of the rat and rabbit eye, mouse mammary gland, mouse melanoma, mouse liver, human and murine epidermis, neurons of the central nervous system and others.

**Description of the Used Lentiviral System**

In our laboratory, lentiviral delivery of cDNAs of interest into cells is currently the preferred method over other delivery systems such as plasmids, retro- and adenoviruses. We are using a lentiviral system produced by the Invitrogen Corporation (Carlsbad, CA). The lentivirus is a VSV-G pseudotype lentivirus based on the HIV-1 vector (strain NL4-3) that recently became commercially available. Packaging, structural, and expressing lentiviral components are separated into four different plasmids (VSVG, pLP1, pLP2 and an Expression vector) in the Invitrogen system to enhance system biosafety. The three plasmids pLP1, pLP2, VSV-G (gag/pol, rev and VSV-G envelope) are designed as packaging plasmids that provide helper components including structural, virus packaging, integration components, as well as lentiviral proteins. VSV-G pseudotype lentiviruses bind to target cells in a receptor-independent manner by using a common phospholipid in the target cell membrane (phosphatidylserine has been proposed as the most probable target). Therefore, these viruses have a broad host range and are most efficient in infecting mammalian cells *in vitro* and *in vivo.*

The expression vector, or entry vector, is used for the delivery of an exogenous cDNA under the CMV promoter. Also, the expression vector is responsible for expression of an antibiotic resistance gene (blasticidin-resistance gene) under the early SV40- promoter. The entry vector can theoretically accommodate up to approximately 6 kb of inserted foreign gene. In our laboratory, we have successfully cloned DNA fragments up to 3.4 kb.

Along with others, we have found that CMV promoter-driven expression is not equivalent in different cell types. CMV promoter does not efficiently regulate expression of introduced genes in NIH3T3 cells and often in other rodent fibroblasts. The CMV promoter can be easily removed using unique restriction sites such as for example, ClaI and BamHI in pLenti6/V5-D-TOPO. Therefore, it may be beneficial to replace CMV promoter with a more efficient or cell type specific promoter. The lentiviral system also allows the introduction of shRNA (short hairpin RNA to silence the gene expression) into the cells.

The lentiviral system we use has a number of built-in safety features that, in combination, make it possible to work within the safety level II requirements. All the vectors lack complete...
gag/pol, envelope, and any accessory genes. Gag/pol are supplied on separate vectors pLP1 and pLP2, respectively. Also, the U3 region in the 3'LTR is deleted so that the integrated virus has no promoter elements in the 5' LTR. Thus, the viruses self-inactivate once they integrate into the host chromosome.

**Technical Outline of Materials and Methods**

**Generation of recombinant lentivirus stocks.** The four lentiviral vectors are transfected into 293FT packaging cells (Invitrogen Corporation) using Lipofectamine2000 or FuGene. 293FT cells stably express large T antigen, which enhances virus packaging and production. The supernatant culture media containing lentivirus may be collected several times during the 2-3 days following transfection. If necessary, the lentivirus can be concentrated by centrifugation at 45,000-50,000 g. Lentiviral stocks are stable at 4°C for 12-24 hrs and can be stored at -80°C for several months. Lentiviral stocks are sensitive to multiple freezing/thawing cycles.

**Evaluation of lentiviral titer and infection efficiency.** The viral titer is determined using the HT1080 human fibrosarcoma cell line (ATCC, Manassas, VA). These cells are monitored using fluorescent microscopy to determine YFP expression. We are currently able to generate viral titers of $10^5$ infectious units per ml (iu/ml).

**Quality and safety control of viral stocks.** Even though the generation of replication-competent virus (RCV) in 293FT cells is a rare event, all lentiviral stocks are examined for the presence of RCV using a sensitive marker rescue assay. This is carried out by monitoring p24 antigen expression in the culture medium. Viral p24 antigen concentration (detection limit = 3 pg/ml) is determined by ELISA (Alliance, DuPont-NEN).

**Results**

Recently we have generated multiple cell cultures infected with lentiviruses. These cells include human prostate epithelial cells (LNCaP and PC3 cell lines) and human transformed keratinocytes (SCC13), which stably express several proteins of interest for our ongoing research. The following cDNAs have been successfully introduced into these cells: glucocorticoid receptor, wild type and dominant negative IkappaB kinase (IKKs), alpha, beta, and epsilon, yellow and red fluorescent proteins (YFP and RFP). In all the cell lines, the initial lentiviral infection yielded in 40-50 percent of infected cells within the total population. Following antibiotic selection the percentage of infected cells was increased to 70-90 percent, depending on the cell line (Figure 1A).

The infected cell lines have been propagated in the presence of selecting antibiotic (blasticidin) for more than 10 months, and the amount of cells expressing the exogenous cDNA has not changed. The initial high infectious capacity of the lentivirus makes it a better choice than retroviral vectors for infecting slowly proliferating adherent cell lines. The high stability of the gene expression and high amount of infected cells are major advantages of lentiviruses over adenoviruses, which do not
integrate into host DNA and provide stable gene expression for a maximum of only 7-10 days.

Another important feature of lentiviral infection is that the level of protein expression provided exogenously is comparable to the amount of endogenous expression. Figure 1B illustrates levels of expression of the exogenous glucocorticoid receptor in prostate carcinoma LNCaP PC cells in comparison to endogenous glucocorticoid receptor in prostate carcinoma cell lines DU145 and PC3. These results demonstrate that lentiviral systems are preferable for generating model cell lines that express proteins within physiological limits.

To track transfected cells in vitro and in vivo, we co-infected cells with both a lentivirus containing the gene of interest and a lentivirus containing YFP as a tracking element. We experimentally found that repeated infection of cells will target the same cell population that incorporated the virus during the first infection (Fig. 2A).

Cell lines stably or transiently expressing a fluorescent protein (YFP or RFP in our laboratory) may be used for a number of experiments such as: assessment of the cell proliferation, evaluation of colony formation in vitro, in vivo tracking of cells injected or transplanted into animals, migration and adhesion assays, and others. Figures 2A, B and C show the examples of proliferation and colony forming assays performed on LNCaP cells expressing the YFP protein. Introduction of the fluorescent proteins may be also used as an alternative to antibiotic based selection of lentiviruses infected clones, as Flow-cytometry may be used to separate positive and negative populations. In our hands, the flow-cytometric selection of cells resulted in an approximately 90 percent pure population of YFP-positive cells.

The lentiviral system can also be successfully used to introduce shRNA into the cells. To introduce shRNA we substituted the CMV promoter for the U6 human specific promoter in the expressing cassette. The preliminary data indicated that the introduction of glucocorticoid receptor shRNA into human

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**Figure 2. Analysis of YFP tracker expression and application of YFP-infected cells for proliferation and colony formation assay.**

A. Co-expression of GR and YFP in cell line. The LNCaP prostate carcinoma cell line was used to establish a line expressing YFP and GR. Cells were first infected with GR-expressing lentivirus and then YFP-expressing lentivirus. A1. Nuclease staining of activated GR (as described in the legend for Fig. 1). A2. YFP expression in LNCaP cells. B. Example of using YFP-expressing cells for assessment of colony formation. LNCaP cells were plated in 1% agarose in cell culture medium. The colonies were evaluated 20 days after plating by Zeiss Axiovert inverted fluorescent microscope. C. Evaluation of cell growth using YFP-expressing cells. YFP-expressing cells were plated in 12 well plates at a concentration of 5000 cell per well and incubated with a cytokine reagent for 1-10 days. C1. Actu1. Images of the YFP expressing cells were taken using the inverted fluorescent microscope by the day 1, 6 and 10 of the treatment with the cytokine reagent. C2. Cell proliferation bar diagram obtained using a fluorescent plate reader.
keratinocyte cell line SCC13 has resulted in the decreased expression and function of endogenous GR (data not shown).

Many years of promising research on lentiviruses has made them one of the most preferable tools for the gene delivery. The lentiviral gene delivery is currently used to deliver genes in vitro and in vivo, for generating transgenic and knockout animals, and obviously has a prospective for the future use in clinic for the treatment of different diseases. However, the ideal gene transfer system is yet to be designed. The different features of lentiviral systems including the efficiency of viral uptake by the cells, controlled integration of viral RNA into the host genome, improved virus tracking, lower viral toxicity, and decreased immune response to the lentiviral infection have to be improved before the lentiviruses will be used in clinical trials. 15

References


Hormonal Regulation of Tumorigenesis
Nancy L. Weigel, Suzanne A. Fugna, and Donald Tindall
Hyatt Regency Monterey • Monterey, California, USA • February 20-25, 2005

Supported by
The Director's Fund
121 Association of CYP2D6 and SULT1A1 polymorphisms and the benefit of tamoxifen treatment in postmenopausal breast cancer patients

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Tamoxifen has in decades been used to treat estrogen receptor positive breast cancers. The anti-tumour effect is related to its ability to antagonise the estrogen receptor signalling pathway and thus suppress the mitogenic effects. The majority of patients initially respond well to the tamoxifen therapy, although a substantial proportion acquire resistance and relapse in the disease by incompletely understood mechanisms. In the present study we propose that tamoxifen resistance may be a result of differences in activity of metabolic enzymes due to genetic polymorphism. Cytochrome P450 2D6 (CYP2D6) and sulfotransferase 1A1 (SULT1A1) are polymorphic and involved in the metabolism of tamoxifen. The CYP2D6*4 and SULT1A1*2 genotypes result in lowered or increased enzyme activity. We investigated the genotypes of CYP2D6 and SULT1A1 in 968 postmenopausal breast cancer patients treated with tamoxifen therapy. The patients were genotyped using PCR followed by cleavage with restriction enzymes. There were no significant differences in distant recurrence-free survival among the genotypes of CYP2D6 and SULT1A1, although a trend of improved benefit in patients harbouring the CYP2D6*4 allele was observed. In conclusion, our results indicate that the metabolic enzymes CYP2D6 and SULT1A1 are not main determinants of tamoxifen resistance.

122 Synergistic effects of nuclear receptor coactivator CoCoA and β-catenin

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The Wnt-signaling cascade plays an important role in developmental processes and is implicated in a variety of cancers. β-catenin is a pivotal component of the Wnt-signaling pathway. It binds to and serves as a primary coactivator for the T Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) family, as well as the nuclear receptor, androgen receptor (AR). Members of the nuclear receptor coactivator complex, including CARM1, p300/CBP, and GRIP1 (one of the p160 coactivators) have been shown to associate with β-catenin and enhance transcriptional activation. We recently identified a factor involved in nuclear receptor-mediated transcriptional activation: the coiled-coil coactivator (CoCoA). CoCoA stimulates transcription by interacting with nuclear receptors through binding to the p160 coactivators. Here we report that CoCoA directly interacts with β-catenin through its N- and C-terminus. This interaction synergistically enhanced transcriptional activation in both AR and TCF/LEF-mediated pathways. Furthermore, while CoCoA associates with both β-catenin and the p160 coactivators, the specific domains required for the cooperation of the coactivators are different. Reduction of endogenous CoCoA level decreased the transcriptional activation of transiently transfected reporter gene controlled by TCF/LEF-responsive enhancer element. The involvement of CoCoA in the Wnt/β-catenin-signaling pathway suggests that the coactivator function of CoCoA is not restricted to the nuclear receptor pathway, but rather, CoCoA may act as a general transcription factor.

123 Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells

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Glucocorticoids are extensively used for combinational chemotherapy of patients with advanced prostate cancer (PC). Due to the widespread use of these hormones, there has been a systematic analysis of the expression and function of glucocorticoid receptor (GR) in PC cells. GR is a well-known transcription factor that belongs to a superfamily of nuclear hormone receptors. We performed immunostaining of more than 50 PC samples (Gleason score 6-10) from untreated patients, and revealed that expression of GR was strongly decreased in epithelial PC cells in comparison to epithelial cells in apparently normal prostate gland and BPH. Interestingly, some PC cell lines including LNCaP cells, lack GR expression. This allows drawing a parallel between those cell lines and PC tumors. To better understand what role GR plays in PC cells, we generated LNCaP cell line stably expressing GR using CMV/GR lentivirus. Treatment of LNCaP-GR cells with synthetic glucocorticoid fluocinolone acetonide (FA) induced growth inhibition of LNCaP-GR cells that correlated with up-regulation of p21 and down-regulation of cyclins D1 and B1. In addition, glucocorticoid treatment significantly increased the expression of genes that have been implicated in PC development. We found that in LNCaP-GR cells treated with FA the expression of muscle and ESE-3 genes characterizing normal prostate were increased. In contrast, the expression of PC markers PSA and PDE5 were decreased in FA-treated cells. This suggests that glucocorticoid treatment induced partial normalisation of PC cell phenotype. We and others showed that NF-κB plays an important role in the resistance of PC cells to apoptosis. Importantly, the restoration of GR expression in LNCaP cells has resulted in inhibition of basal and inducible NF-κB activity, and activation of some pro-apoptotic genes. Taken together these data suggest that GR may act as tumor suppressor in prostate through control of expression of genes involved in normal prostate differentiation, growth and apoptosis.

Work was supported by DOD prostate grants DAMD17-01-1-0015 and DAMD17-03-1-0522 and Northwestern University Prostate SPORE Developmental Project.

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Withdrawn

Hormonal Regulation of Tumorigenesis
Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines.

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Prostate cancer (PC) is the second leading cause of death among cancers in men. One of the contributing factors to high mortality rate from PC is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in PC cells could play a strategic role for PC treatment. One of the central mechanisms protecting cells from apoptotic death is mediated by NF-kappaB factors that control the expression of numerous anti-apoptotic genes. We and others showed previously that NF-kappaB transcription factor was constitutively active in PC cell lines and in human prostate tumors due to the up-regulated activity of IkappaB-kinases (IKK), mostly IKK-beta. In this work we investigated effect of a novel highly specific IKK-beta inhibitor PS1145 on constitutive and inducible NF-kappaB activity in human cell lines PC-3 and DU145 using Luciferase Assay with x5.kappaB-Luciferase reporter, EMSA, Northern blot analysis of expression of endogenous kappaB-responsive genes, Western blot analysis of IkappaB alpha phosphorylation, degradation and p65 nuclear translocation. Our studies revealed that PS1145 at the dose range 5-20 μM efficiently inhibited both basal and induced by either TNF-alpha or LPS NF-kappaB activity in PC cells. PC3 and DU145 cells are known to be resistant to TNF-alpha-induced apoptosis partially due to the constitutively active NF-kappaB. We found that PS1145 significantly sensitized PC cell lines to TNF-alpha induced apoptosis. We observed the elevated PARP cleavage and caspase 3/7 activation when cells exposed to TNF-alpha were pretreated with PS1145. Currently we are evaluating the expression of kappaB-responsive genes as well as PC gene markers in prostate cells upon PS1145 treatment in vitro and in vivo. Supported by DOD prostate cancer research grants DAMD17-01-1-0015 and DAMD17-03-1-0522.