

1 α ,25(OH)₂-VITAMIN D₃ IN PROSTATE:
INTERSECTION WITH AKT/PTEN AXIS AND ROLE IN SENESCENCE

BY

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(A major portion of this chapter was published in the *Prostate*, June 2010)

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LIST OF ABBREVIATIONS

1 α ,25(OH) ₂ D ₃	- 1-alpha, 25-dihydroxy-vitamin D ₃
25(OH)D ₃	- 25-dihydroxy-vitamin D ₃
AIPC	- androgen-independent prostate cancer
ANOVA	- analysis of variance
ARF	- alternate reading frame
ARR	- androgen-responsive region
ARR2PB-Cre	- Cre-recombinase under probasin promoter containing two AARs
ATP	- adenosine triphosphate
BAD	- BCL2-associated agonist of cell death
BAX	- BCL2-associated X protein
BCL2	- B-cell CLL/lymphoma 2
BID	- BH3 interacting domain death agonist
CDK	- cyclin-dependent kinase
CDK2	- cyclin-dependent kinase 2
CDKN1A	- cyclin-dependent kinase inhibitor 1A gene (encodes p21)
CHK1	- checkpoint kinase 1
CI	- combination index or confidence interval
CKIs	- cyclin-dependent kinase inhibitors
CKS-1	- cyclin-dependent kinases regulatory subunit 1
COX-2	- cyclooxygenase-2
CYP24A1	- cytochrome P450, family 24, subfamily A, polypeptide 1 gene (encodes 24-hydroxylase)
CYP27B1	cytochrome P450, family 27, subfamily b, polypeptide 1 gene (encodes 1 α -hydroxylase)

DBP	vitamin D transporter protein
DMSO	- dimethyl sulfoxide
DRE	- digital rectal examination
DRI	- dose-reduction index
DSB	- double-strand breaks
DTT	- dithiothreitol
E μ -myc	- c-myc under control of the immunoglobulin heavy chain enhancer
ECM	- extracellular matrix
EDTA	- ethylenediaminetetraacetic acid
EGF	- epidermal growth factor
EGFR	- epidermal growth factor receptor
FBS	- fetal bovine serum
FGF	- fibroblast growth factors
FGFR1	- fibroblast growth factor receptor 1
FOXO	- forkhead box O
GSK-3	- glycogen synthase kinase 3
HDAC1	- histone deacetylase 1
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HL-60	- human promyelocytic leukemia cell line
HP1 γ	- heterochromatin protein 1 gamma
HPV	- human papilloma virus
HR	- hazard ratio
IC ₅₀	- half maximal inhibitory concentration
IFG-1R	- insulin-like growth factor-1 receptor
IGF	- insulin-like growth factor

IGFBP-3	- insulin-like growth factor binding protein 3
IL-6	- interleukin 6
IL-8	- interleukin 8
IU	- international unit
LOH	- loss of heterozygosity
LSD	- least significant difference
MDM2	- murine double minute 2
MEFs	- mouse embryonic fibroblasts
MMAC1	- mutated in multiple advanced cancers
MMP-2	- metalloproteinase 2
MMP-9	- metalloproteinase 9
MMTV	- mouse mammary tumor virus
MPEC	- mouse prostatic epithelial cells
mTOR	- mammalian target of rapamycin
mTORC2	- mammalian target of rapamycin complex 2
NCI	- National Cancer Institute
NF-1	- Neurofibromatosis type I
NP	- nonyl-phenoxypolyethoxylethanol
NSAIDs	- nonsteroidal anti-inflammatory drugs
PB	- probasin
PBS	- phosphate buffered saline
PCa	- prostate cancer
PCR	- polymerase chain reaction
PDGF	- platelet-derived growth factor
PKD1	- phosphoinositide-dependent kinase 1

PDZ	- <u>P</u> SD-95, <u>D</u> iscs-large, <u>Z</u> O-1
PEST	- proline- (P), glutamate- (E), serine- (S), and threonine-(T)-rich sequence
PH	- plekstrin homology domain
PI-3,4,5-P3	- phosphatidylinositol 3,4,5-triphosphate
PI-3,4-P2	- phosphatidylinositol 3,4-biphosphate
PI3K	- phosphoinositol-3 kinase
PIN	- prostatic intraepithelial neoplasia
PKB	- protein kinase B
PML	- promyelocytic leukemia
PMSF	- phenylmethylsulfonyl fluoride
Pol II	- RNA polymerase II
PP1	- protein phosphatase 1
PP2A	- protein phosphatase 2A
pRb	- retinoblastoma protein
PSA	- prostate specific antigen
PSADT	- PSA doubling time
PTEN	- <u>p</u> hosphatase and a <u>t</u> ensin homologue deleted in chromosome <u>ten</u>
PTKs	- protein-tyrosine kinases
PVDF	- polyvinylidene difluoride
Rb	- retinoblastoma
RHEB	- Ras homolog enriched in brain
RXR	- retinoid X receptor
SAHF	- senescence-associated heterochromatin foci
SA- β -gal	- senescence-associated β -galactosidase activity
SCF	- Skp, Cullin, F-box containing complex

SD	- standard deviation
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	- standard error
shRNA	- short hairpin RNA
Skp2	- S-phase kinase-associated protein 2
SV40	- simian virus 40
TCN-PM	- triciribine phosphate monohydrate
TDEC	- tumor-derived endothelial cells
Tg-AKT1	- transgenic constitutively active (myristoylated) AKT1
TGF- β	- transforming growth factor- β
TK1	- thymidine kinase 1
TRAMP	- transgenic adenocarcinoma mouse prostate
Tris	- tris(hydroxymethyl)-aminomethane
TSC2	- tuberous sclerosis complex 2
TYMS	- thymidylate synthetase
UVB	- ultra-violet B
v/v	- volume to volume
VDR	- vitamin D receptor
VDRE	- vitamin D response elements
Vhl	- von Hippel-Lindau
γ H2AX	- histone H2AX phosphorylated at Ser 139 position

ABSTRACT

Axanova, Linara Shamilevna

1 α ,25(OH) $_2$ -VITAMIN D $_3$ IN THE PROSTATE: INTERSECTION WITH THE AKT/PTEN AXIS AND ROLE IN SENESENCE

Dissertation under the direction of Scott D. Cramer, Ph.D., professor of Cancer Biology

The PI3K-AKT pathway is frequently activated in prostate cancer and can lead to downregulation of p21 and p27 levels and function. 1-alpha, 25-dihydroxy vitamin D $_3$ (1 α ,25(OH) $_2$ D $_3$) inhibits proliferation of multiple cancer cell types, including prostate cancer cells, and upregulates p21 and/or p27. We hypothesized that inhibition of the PI3K/AKT pathway would synergize with the antiproliferative signaling of 1 α ,25(OH) $_2$ D $_3$. We found that pharmacological inhibitors of PI3K or AKT and 1 α ,25(OH) $_2$ D $_3$ synergistically inhibit growth of DU145, LNCaP and primary human prostate cancer cells. Combination of 1 α ,25(OH) $_2$ D $_3$ and an AKT inhibitor cooperated to induce G $_1$ arrest, senescence and higher p21 levels in prostate cancer cells. As AKT activation in prostate cancer usually occurs due to the loss of tumor suppressor PTEN, we evaluated the effect of Pten loss on 1 α ,25(OH) $_2$ D $_3$ -mediated antiproliferative effects utilizing mouse prostatic epithelial cells (MPEC) with acute *in vitro* Pten knockdown or knockout. We found that loss of Pten expression led to a partial induction of cellular senescence. Upon treatment with 1 α ,25(OH) $_2$ D $_3$, MPEC which had lost Pten showed significantly higher levels of growth inhibition and senescence induction compared to the Pten-expressing counterparts. In contrast to the MPEC with *in vitro* acute deletion of Pten, *Pten null* MPEC isolated from Pten-deletion-driven *tumors* showed a complete loss of sensitivity to 1 α ,25(OH) $_2$ D $_3$ -mediated growth inhibition, which was partially restored by co-treatment with a

PI3K or AKT inhibitor. Moreover, this combination led to synergistic growth inhibition of tumor-derived *Pten null* MPEC. These observations suggest that initial loss of Pten in prostate cells may result in an increased sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated antiproliferative effects, possibly by additive effect on senescence induction; whereas subsequent changes associated with tumor progression may lead to abrogation of $1\alpha,25(\text{OH})_2\text{D}_3$ -sensitivity, which at least partially can be overcome by combination with PI3K/AKT inhibitors. We have also discovered a novel mechanism for the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ - senescence. Together, these findings providing a rationale for further testing of therapeutic intervention using $1\alpha,25(\text{OH})_2\text{D}_3$ for slowing the process of tumorigenesis of earlier pre-tumoral stages of prostate neoplastic disease as well for testing combinations of $1\alpha,25(\text{OH})_2\text{D}_3$ with PI3K/AKT inhibitors for the treatment of prostate cancer.

CHAPTER I

GENERAL INTRODUCTION

1.1. Prostate Cancer Statistics and Disease Progression

Adenocarcinoma of the prostate gland is the second most commonly diagnosed malignancy in American men, following skin cancer. In 2010 alone, over 217, 700 new cases of prostate cancer will be diagnosed and about 32,050 men will die of prostate cancer, according to the latest American Cancer Society estimates (1). Prostate cancer (PCa) accounts for about 1 in every 4 newly diagnosed cancers among US men, with overall prognosis predicting 1 man in 6 to be diagnosed with this disease during his lifetime. Although PCa is generally a slow growing malignancy, only lung cancer accounts for more cancer deaths among U.S. men. Since PCa rates increase with age, it can be expected that PCa would become an even greater problem as life expectancy continues to increase, presenting a major public health concern in the U.S. and worldwide.

Early diagnosis of PCa is often made by screening for prostate specific antigen (PSA), as well as digital rectal examination (DRE). If the cancer is confined to the prostate, therapeutic choices are prostatectomy or radiation. In elderly men with less invasive pathology on biopsy or those with comorbidities, 'watchful waiting' with monitoring PSA levels is often suggested. If the cancer has already escaped the capsule, androgen ablation is the first-line treatment leading to significant apoptosis of prostatic cancer cells. However, within 18-24 months after androgen ablation, the majority of patients relapse with androgen-independent prostate cancer (AIPC) (2). The median survival once AIPC develops is 12 to 18 months. Thus, therapies that can prevent PCa, prolong the hormone-dependent state, or are effective against androgen-independent prostate cancer are urgently needed.

The etiologic factors that may be associated with PCa include age, race, as well as genetic, dietary, and hormonal influences (3,4). Environmental factors may also have an influence on the

disease expression. For example, it has long been recognized that solar radiation can decrease the mortality rates of noncutaneous malignancies (5,6).

In 1990 Schwartz and Hulka suggested a role for vitamin D in decreasing the risk of developing prostate cancer based on the observation that prostate mortality rates in the U.S. are inversely proportional to the geographically determined UV radiation exposure from the sun, and the fact that UV light is essential for vitamin D synthesis (7). Since the initial hypothesis 30 years ago, a great number of pre-clinical and clinical studies evaluated the potential of vitamin D₃ and its metabolites in prevention and treatment of prostate cancer, as discussed below.

1.2. Vitamin D

1.2.1. Vitamin D Discovery

Groundbreaking discoveries of the early 20th century elucidated the essential role of vitamin D₃ in the metabolism of calcium and phosphorus and, consequently, bone mineralization. These findings provided a major public health advance, allowing for a nearly complete eradication of rickets in children in the US.

Rickets is a disease of children of either rich families deliberately avoiding exposure to sunshine in countries such as United Kingdom and India, or very poor families living in the slums of big European industrialized cities. The seminal studies by Mellanby (8) and Chick et al. (9) shed light on the origin of the disease, and demonstrated that clinical rickets can be cured by dietary cod liver oil supplementation or sunlight exposure, both of which result in increased vitamin D₃ levels. In 1928, the Nobel prize was awarded to Dr. Adolf Windaus, in recognition of his achievements in identifying of the chemical structure and achieving the chemical synthesis of Vitamin D. In the 1930s, fortification of milk with vitamin D₃, increased recognition of the importance of sunshine exposure and cod liver oil supplementation virtually eradicated rickets

from the United States, although it had previously been a highly prevalent crippling disease of childhood (10).

1.2.2. Chemical Structure of $1\alpha,25(\text{OH})_2\text{D}_3$

$1\alpha,25(\text{OH})_2\text{D}_3$, the active form of vitamin D_3 , is a highly flexible molecule with a steroid carbon skeleton, involving 4 fused cyclopentanoperhydro-phenanthrene rings, A-D (Figure 1). Unlike other steroids, the 9-10 carbon bond is broken, creating a conformationally flexible molecule in which ring A may rotate; thus, the molecule is technically classified as a seco-steroid. These special features of the molecule play an important role in its biological function, with *cis-trans* isomerism effecting its stability and reactivity, while the unusual degree of flexibility of the molecule enables synthesis of structural analogs.

1.2.3. Vitamin D Metabolism

Vitamin D is unique compound in that while being a vitamin that can be obtained from food, it is also a hormone that can be synthesized in the body upon exposure to UV-B light.

The mammalian form of vitamin D_3 is a fat-soluble prohormone cholecalciferol (vitamin D_3) that may be generated endogenously by UV light-mediated metabolism of the precursor 7-dehydrocholesterol in the skin (Figure 2). This form of vitamin D_3 , as mentioned earlier, can be obtained from dietary sources (11). Cholecalciferol (vitamin D_3) is hydroxylated to $25(\text{OH})$ -vitamin D_3 ($25(\text{OH})\text{D}_3$) by hepatocyte 25-hydroxylase. Further hydroxylation by 1α -hydroxylase (encoded by CYP27B1 gene) into main biologically active hormone, $1\alpha, 25(\text{OH})_2$ -vitamin D_3 ($1\alpha,25(\text{OH})_2\text{D}_3$, also known as calcitriol) occurs in proximal renal tubules in a tightly regulated fashion (12). $1\alpha,25(\text{OH})_2\text{D}_3$ levels are precisely controlled by a feed-back regulation loop. High levels of $25(\text{OH})\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ induce the expression of 24-hydroxylase (encoded by the gene CYP24A1) which catabolizes both $25(\text{OH})\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$, ultimately leading to their

B.

The structure shows a bicyclic system consisting of a cyclohexane ring fused to a cyclopentane ring. The cyclohexane ring has a methyl group at the bridgehead position (C1) and a side chain at the adjacent position (C2). The side chain is a 4-hydroxy-4-methylpentyl group, shown with a dashed bond to the cyclohexane ring and a wedged bond to the methyl group. The cyclopentane ring has a double bond between C5 and C6, and a methyl group at C5. A separate cyclohexene ring is shown below, with a double bond between C1 and C2, and a methyl group at C3. The cyclohexene ring has a hydroxyl group at C4 (wedged bond) and a methyl group at C5 (wedged bond). A curved arrow indicates the movement of electrons from the double bond in the cyclohexene ring to the double bond in the bicyclic system, suggesting a conjugative or resonance effect.

4

inactivation and secretion (13,14). Aside from renal tubules, bone, brain, skin, colon, breast and prostate cells were shown to express the 1α -hydroxylase, enabling them to convert the major circulating metabolite of vitamin D₃, $25(\text{OH})\text{D}_3$ to $1\alpha,25(\text{OH})_2\text{D}_3$ and locally regulate the levels of $1\alpha,25(\text{OH})_2\text{D}_3$ (15).

$1\alpha,25(\text{OH})_2\text{D}_3$ is the most active form of vitamin D and acts as a steroid chemical messenger in multiple target tissues which make up the so-called “vitamin D endocrine system” (16). $1\alpha,25(\text{OH})_2\text{D}_3$ plays a critical role in regulation of serum calcium and phosphorus levels and bone mineralization by stimulating intestinal calcium and phosphorus absorption, renal calcium and phosphorus reabsorption, as well as by differential effects on osteoblasts and chondrocytes (Figure 2) (17). Expression of multiple elements of the vitamin D endocrine system in diverse human tissues demonstrated further complexity and importance of vitamin D₃ signaling beyond bone homeostasis (16). Most tissues express the receptor for $1\alpha,25(\text{OH})_2\text{D}_3$, the vitamin D receptor (VDR), while renal tubules, bone, brain, skin, colon, breast and prostate were shown to express the 1α -hydroxylase (CYP27B1), required for conversion of the major circulating metabolite of vitamin D₃, $25(\text{OH})\text{D}_3$ to $1\alpha,25(\text{OH})_2\text{D}_3$ (15).

1.2.4. Vitamin D Transport

Less than 1% of $25(\text{OH})\text{D}_3$ or $1\alpha,25(\text{OH})_2\text{D}_3$ is free in plasma, with the remainder being bound to vitamin D transporter protein (DBP) (85-88%; high affinity) (18) or albumin (12-15%; low affinity) (19). In addition to transport, DBP maintains serum stores of vitamin D metabolites and modulates bioavailability (20). Notably, only free unbound vitamin D molecules are considered to be biologically active (21).

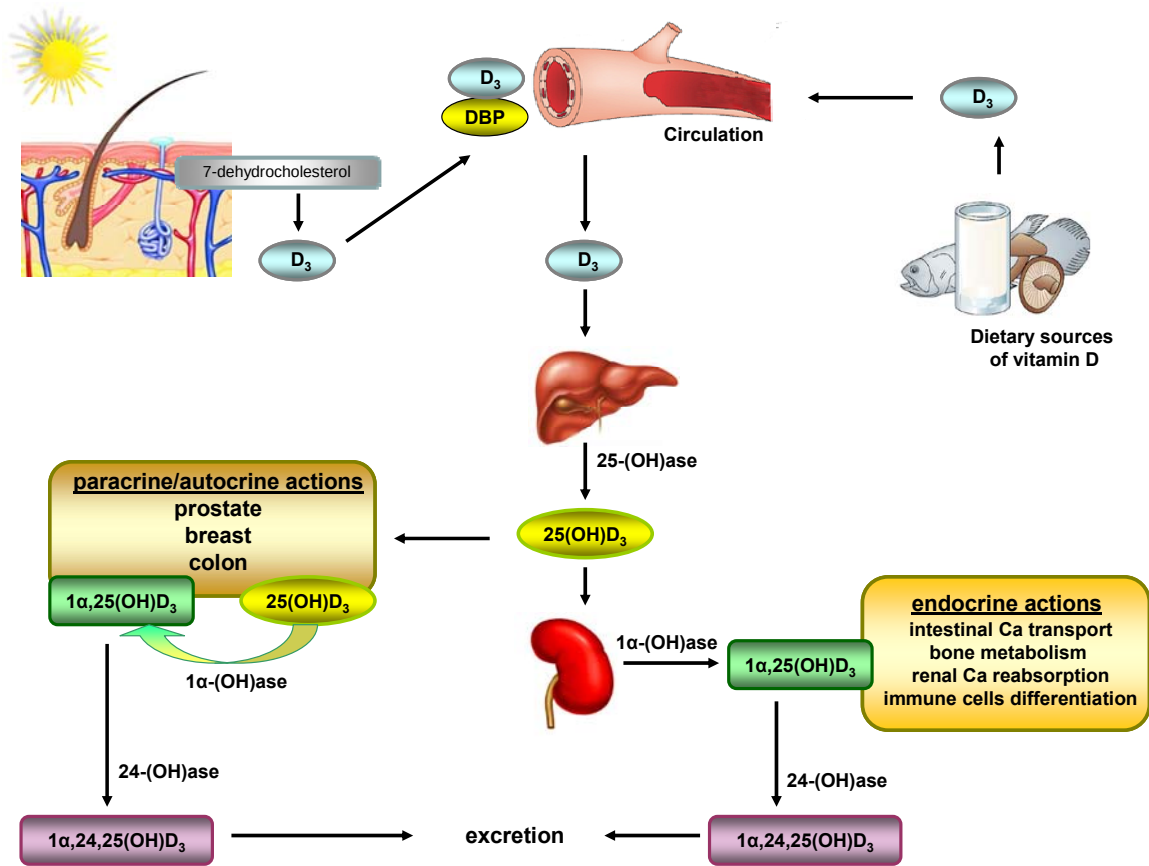


Figure 2. Vitamin D metabolism. Synthesis of $1\alpha,25(\text{OH})_2\text{D}_3$ starts with photochemical conversion of 7-dehydrocholesterol to pre-vitamin D_3 (pre- D_3) in response to UVB exposure in the skin. Vitamin D_3 , obtained from the isomerization of pre-vitamin D_3 in the epidermal basal layers or intestinal absorption of natural and fortified foods and supplements, binds to vitamin D-binding protein (DBP) in the bloodstream, and is transported to the liver. Vitamin D_3 is hydroxylated by liver 25-hydroxylases (25-(OH)ase). The resultant $25(\text{OH})\text{D}_3$ is 1α -hydroxylated in the kidney by 25-hydroxyvitamin D_3 - 1α -hydroxylase (1α -(OH)ase). This yields the active secosteroid $1\alpha,25(\text{OH})_2\text{D}_3$ (calcitriol), which has different effects on various target tissues. The rate-limiting step in catabolism is the degradation of $25(\text{OH})\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ to $24,25(\text{OH})_2\text{D}_3$ and $1\alpha,24,25(\text{OH})_3\text{D}_3$, respectively, which occurs through 24-hydroxylation by 25-hydroxyvitamin D 24-hydroxylase (24-(OH)ase). $24,25(\text{OH})_2\text{D}_3$ and $1\alpha,24,25(\text{OH})_3\text{D}_3$ are consequently excreted.

1.2.5. $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated Transcription of the Target Genes

The classical genomic action of $1\alpha,25(\text{OH})_2\text{D}_3$ involves the regulation of transcription of target genes. $1\alpha,25(\text{OH})_2\text{D}_3$ exerts transcriptional activation and repression of target genes by binding the VDR that may be present in the cytoplasm, the nucleus or partitioned between the cytoplasm and the nucleus (22). $1\alpha,25(\text{OH})_2\text{D}_3$ -bound VDR forms heterodimers with any of the three isoforms of the retinoid X receptor (RXR), and these dimers occupy specific binding sites on DNA (vitamin D response elements (VDRE)). Upon recruitment of other coregulatory proteins, this complex induces transcription of vitamin D responsive genes (23) (Figure 3).

Transcriptional targets of ligand-bound VDR show very high degree of tissue-, cell-, and developmental stage-specificity. VDR expression has been identified in virtually every human tissue, albeit at different concentrations (reviewed in (12, 24)), and its signaling affects hormone secretion, immune function, cell differentiation, proliferation and growth (reviewed in (11)).

1.3. Role of Vitamin D in Prostate Cancer

1.3.1. Epidemiological Studies on Vitamin D and Prostate Cancer

Circulating levels of $25(\text{OH})\text{D}_3$ account for all sources of vitamin D (including nutritional supply as well as conversion of vitamin D into $25(\text{OH})\text{D}_3$), and circulating $25(\text{OH})\text{D}_3$ has a relatively long half-life (2 to 3 weeks), providing a stable indicator of long-term vitamin D status. In addition, levels of $25(\text{OH})\text{D}_3$ in the serum are important, as $1\alpha,25(\text{OH})_2\text{D}_3$ is readily synthesized from it by the 1α -hydroxylase enzyme that is ubiquitous in epithelial tissues of multiple organ tissues, including prostate (25).

Overall, most epidemiological studies have reported that higher serum $25(\text{OH})\text{D}_3$ levels are associated with lower incidence rates of various cancers including colon (26-27), breast (28-30) and ovaries (31). The results of studies evaluating prostate cancer and vitamin D_3 status

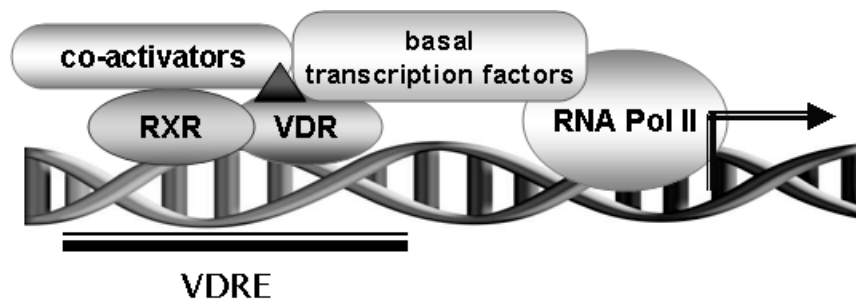


Figure 3. $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated transcriptional regulation. Upon binding of $1\alpha,25(\text{OH})_2\text{D}_3$ to VDR, the $1\alpha,25(\text{OH})_2\text{D}_3$ -VDR complex heterodimerizes with RXR, and the VDR/RXR heterodimer binds VDREs in the promoter regions of the target gene. Transcriptional activation requires the action of many multisubunit coactivator complexes that are recruited in a parallel and/or sequential manner. This assembly of proteins then attracts components of the RNA polymerase II (Pol II) preinitiation complex and nuclear transcription regulators, thereby altering the rate of gene transcription.

associations are providing less consistent results, with some studies showing higher 25(OH)D₃ levels to be associated with lower risk of prostate cancer or less aggressive disease development (32-34), while results of other studies do not support such an association (35-38).

Several factors could contribute to the apparently inconsistent findings in evaluating the association between vitamin D status and prostate cancer incidence. First, vitamin D status could be more relevant for prediction of disease progression rather than for overall prostate cancer risk. For instance, recent study following up about 15,000 men for 18 years demonstrated that men with low levels of both 25(OH)D₃ and 1 α ,25(OH)₂D₃ had an increased risk for aggressive (advanced stage or high-grade) prostate cancer while 25(OH)D₃ and 1 α ,25(OH)₂D₃ were not good predictors of non-aggressive prostate cancer (34).

Second, the dose-response relation between vitamin D levels and prostate cancer risk may be operative at quite low levels of 25(OH)D₃. For example, in populations with prolonged severe vitamin D deficiency (e.g. Nordic countries where prevalence of vitamin D deficiency is high due to the high latitudes) there is an evidence for an inverse association. Thus, in a Norway study the case-fatality rate of prostate cancer patients with high serum 25(OH)D₃ (>80 nmol) was only one sixth of the case-fatality rate of patients with low serum 25(OH)D₃ (<50 nmol) (odds ratio 0.16, 95% CI 0.05-0.43, p<0.001) (39). A correlation between PCa risk and low serum levels of 25(OH)D₃ has been shown by a case-control study involving 19,000 Finish men followed for 13 years (32). On the other hand, studies evaluating 25(OH)D₃ serum levels in the residents of Hawaii and California reported a lack of an association between low 25(OH)D₃ concentrations and an increased risk for prostate cancer (36, 40). Moreover, a study of men in Hawaii and California suggested an increased risk of prostate cancer with higher concentrations of plasma 25(OH)D₃ (OR for 50ng/ml=1.52, 95% CI=0.92-2.51) (40).

Another reason for the inconsistent results on prostate cancer risk and vitamin D status association might be attributed to the variable and typically prolonged natural history of the disease development that is often measured in decades (41). The process of prostate cancer

tumorigenesis is likely to begin earlier in life (with microscopic neoplastic lesions already prevalent in the prostate gland observed as early as the third decade (42)). In addition, prostate cancer cells may lose the ability of normal prostate epithelial cells to convert 25(OH)D₃, the more prevalent circulating form, to its more active form 1 α ,25(OH)₂D₃ (43,44). Thus, evaluation of vitamin D levels over extended periods of time in a large number of subjects might provide more accurate results. Studies that used longer median periods for follow-up (over 10 years) are more suggestive of a role of vitamin D₃ status in the development of prostate cancer (32-34).

A nested case-controlled study based on a 13-year follow-up of about 19,000 men in Finland found that low levels of 25(OH)D₃ were associated with an increased risk for earlier occurrence and more aggressive development of prostate cancer, especially before the andropause (32). Men with 25(OH)D₃ concentrations below the median had adjusted relative risk (OR) of 1.7 compared to men with 25(OH)D₃ levels above the median. The prostate cancer risk among younger men (< 52 years) at entry and low serum 25(OH)D₃ was higher (OR 3.1 nonadjusted and 3.5 adjusted), and among those younger men, low 25(OH)D₃ entailed a higher risk of non-localized cancers (OR 6.3) (32). In another long-term study, in which over 250,000 serum samples were collected in the 1960s, levels of 25(OH)D₃ and 1 α ,25(OH)₂D₃ were evaluated in the subgroup of men diagnosed with prostate cancer before the end of 1987, and in controls individually matched on age, race, and day of serum storage. Risk of prostate cancer decreased with higher levels of 1 α ,25(OH)₂D₃, especially in men with low levels of 25(OH)D₃, while mean 25(OH)D₃ was not significantly different in cases and controls. The association of lower 1 α ,25(OH)₂D₃ with prostate cancer was found in men above the median age of 57 years at serum storage but not in younger men, and was similar in black and white men (33).

A more recent study by Li and colleagues carried out at Brigham and Women's Hospital followed 14,916 initially prostate-cancer free men for 18 years in the Physicians' Health Study cohort (34). The plasma levels of 25(OH)D₃ and 1 α ,25(OH)₂D₃ were evaluated twice a year (winter/spring and summer/fall). Notably, this study evaluated VDR polymorphisms of the

participants. Over the period of study, 1,066 men developed prostate cancer, including 496 with aggressive disease. The results demonstrated that men with 25(OH)D₃ and 1,25(OH)₂D₃ levels both below the median (25(OH)D₃ of 28ng/mL (70nmol/L) and 1α,25(OH)₂D₃ of 32pg/mL (77 pmol/L)) had twice the incidence of aggressive prostate cancer (odds ratio 2.1, 95% CI 1.2-3.4, p<0.05) compared to men with level above the median (34).

Finally, it has been suggested that vitamin D status earlier in life could play a role in prostate cancer development. John and colleagues demonstrated a significant inverse association between prostate cancer incidence later in life in men born in a region of high solar radiation versus low solar radiation (relative risk of 0.49, 95% CI: 0.27-0.90), with a slightly greater risk reduction for fatal than for nonfatal prostate cancer (45,46).

Taken together, it can be suggested that maintenance of adequate serum vitamin D levels over one's life time should be advised for the reduction of prostate cancer risk. Yet optimal levels of 25(OH)D₃, the length of time required to observe positive effects and the time period of life when exposure is most relevant for prevention of malignancy still remain to be determined.

1.3.2. 1α,25(OH)₂D₃ in Prostate Cancer: *in vitro* and *in vivo* Studies

1α,25(OH)₂D₃ has been shown to inhibit proliferation of cancer cell lines derived from breast (47), lung (48), endometrium (49), head and neck (50), hematopoietic lineages (51) and prostate (52).

The ability of 1α,25(OH)₂D₃ to inhibit prostate growth was demonstrated in primary prostatic cells from histologically normal, benign prostatic hyperplasia, and prostate cancer specimens (53), in multiple prostate cancer cell lines (54-56), in xenograft models of prostate cancer (57-58), as well as in the Dunning rat prostate model (59). The mechanisms of these effects are not completely characterized but include inhibition of: (i) cell proliferation (e.g. through cell cycle arrest) (60), (ii) invasions (61), (iii) migration (62), (iv) metastasis (63-64); and (v) angiogenesis (65).

Growth Arrest. In many cancer cells, treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs results in accumulation of cells in the G0/G1 phase of the cell cycle (66). $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to induce cell cycle arrest by multiple mechanism with cyclin-dependent kinase (CDK) inhibitors p21 and p27 being common targets for $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth arrest. Thus, in LNCaP cells, $1\alpha,25(\text{OH})_2\text{D}_3$ mediated G1 arrest by increasing the expression of p21 and decreasing cyclin-dependent kinase 2 (CDK2) activity, followed by subsequent decrease in the levels of phosphorylated retinoblastoma protein (pRb) and suppression of E2F transcriptional activity (67). CDKN1A (encoding p21) contains a VDRE and is a direct transcriptional target of VDR that has been shown to be directly upregulated by $1\alpha,25(\text{OH})_2\text{D}_3$ in some systems (68). In LNCaP, however, the regulation of p21 appeared to be indirect (67, 69). Boule et al. showed that induction of the insulin-like growth factor binding protein 3 (IGFBP-3) gene by $1\alpha,25(\text{OH})_2\text{D}_3$ resulted in increased p21 protein levels in LNCaP cells and the up-regulation of IGFBP-3 was necessary for the inhibition of cell growth (70). Notably, the IGFBP-3 gene contains a characterized a VDRE in its promoter region (71). In another prostate cancer cell line, ALVA-31, $1\alpha,25(\text{OH})_2\text{D}_3$ also increased p21 mRNA and protein levels (72). Stable transfection of these cells with a p21 antisense construct abolished $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition, demonstrating an essential role of p21 induction in the antiproliferative qualities of $1\alpha,25(\text{OH})_2\text{D}_3$ in these cells. In PC3 prostate cancer cells $1\alpha,25(\text{OH})_2\text{D}_3$ did not increase expression of p21, which is consistent with the lack of G1 accumulation following $1\alpha,25(\text{OH})_2\text{D}_3$ treatment in these cells (73).

p21 is a known p53 target gene (74), however, p53 was not required to induce growth inhibition or G1 arrest induction by $1\alpha,25(\text{OH})_2\text{D}_3$ in LNCaP cells (75). Nevertheless, elimination of p53 function allowed the cells to recover from the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth arrest in LNCaP cells, and eliminated the growth inhibitory effects of combinations of 9-cis retinoic acid and $1\alpha,25(\text{OH})_2\text{D}_3$ (75).

Unlike p21, p27 does not contain VDRE in its promoter, and induction of p27 levels by $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cancer cells occurs through inhibition of p27's proteolysis (76,77).

Thus, upregulation of p27 protein by $1\alpha,25(\text{OH})_2\text{D}_3$ in LNCaP was mediated by downregulation of transcriptional expression of p45Skp2 (S-phase kinase-associated protein 2), the F-box protein which is implicated in p27 degradation (76, 78). $1\alpha,25(\text{OH})_2\text{D}_3$ induced the formation of VDR/Sp1 complex and acted via a Sp1- and HDAC1-dependent pathway to inhibit p45Skp2 promoter activity, which resulted in lower Skp2 levels and reduced turnover of p27^{Kip1} protein (76). In addition, Yang and colleagues (78) showed that $1\alpha,25(\text{OH})_2\text{D}_3$ reduced nuclear levels of CDK2 with consequent reduction of CDK2-mediated phosphorylation of p27 at Thr187 which targets p27 for SKP2-mediated degradation (79,80). The observation of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated decrease in degradation of p27 in prostate cancer cells was confirmed in the other cell types. Thus, $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs can increase levels of both p21 and p27 by decreasing SKP2 levels in thyroid carcinoma (69, 81), and through inhibition of cyclin-dependent kinases regulatory subunit 1 (Cks1), which is another member of SKP2 complex playing a role in the degradation of p27 in promyelocytic leukemia cells (82). Taken together, it was concluded that $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated p27 up-regulation results from increased p27 protein half-life.

In cell culture systems of other cancers types, $1\alpha,25(\text{OH})_2\text{D}_3$ was also shown to act through various mechanisms: in colon cancer, $1\alpha,25(\text{OH})_2\text{D}_3$ was shown to repress TYMS (encoding thymidylate synthetase) and TK1 (encoding thymidine kinase), which are involved in DNA replication (83); in HL60 cells, $1\alpha,25(\text{OH})_2\text{D}_3$ activated the INK4 family of cyclin D-dependent kinase inhibitors (84); in ovarian cancer cells, $1\alpha,25(\text{OH})_2\text{D}_3$ downregulated cyclin E-CDK2 and the Skp2 ubiquitin ligase, which targets cyclin-dependent kinase inhibitors (CKIs) to the proteosome (85-86), and repressed proto-oncogene MYC (87). Thus, the regulation of cell cycle distribution by $1\alpha,25(\text{OH})_2\text{D}_3$ appears to be cell-specific and may involve distinct pathways of action.

Apoptosis. Induction of apoptosis by $1\alpha,25(\text{OH})_2\text{D}_3$ is not uniformly seen in all cancer cells. In the case of PCa, LNCaP is the cell line that some $1\alpha,25(\text{OH})_2\text{D}_3$ -induced apoptosis was observed in upon prolonged treatment (88,89), with only a small fraction of cells demonstrating

its induction (90). Thus, induction of apoptosis by $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cancer cell lines appears to be cell-specific, as it is not commonly observed, with the major anti-proliferative action of $1\alpha,25(\text{OH})_2\text{D}_3$ being the cell cycle arrest.

Differentiation. $1\alpha,25(\text{OH})_2\text{D}_3$ was shown to induce differentiation in multiple normal and malignant cells, including hematopoietic progenitor cells, isolated leukemia cells, colon cancer cells and others (reviewed in (91)). In the PCa cells, LNCaP, PC-3 and MDA PCa 2a and 2b, $1\alpha,25(\text{OH})_2\text{D}_3$ increases the expression of PSA (56, 92), which is considered to be a marker of epithelial prostate cell differentiation. However, strong evidence supporting $1\alpha,25(\text{OH})_2\text{D}_3$ -induced differentiation in prostate cells is still lacking.

Inhibition of Invasion and Metastasis. Several *in vitro* models of prostate cancer suggested that $1\alpha,25(\text{OH})_2\text{D}_3$ might have an ability to reduce tumor invasion and metastasis. Thus, $1\alpha,25(\text{OH})_2\text{D}_3$ and its analog 1,25-dihydroxy-16-ene-23-yne-cholecalciferol inhibited invasiveness of DU145 through Amgel and decreased matrix metalloproteinase-2 (MMP-2) and MMP-9 secretion (61). $1\alpha,25(\text{OH})_2\text{D}_3$ reduced cell adhesion, invasiveness and migration of DU145 and PC3 cells in another study, in part due to decreased expression of α_6 and β_4 integrines (62). In LNCaP and PC-3 cells, $1\alpha,25(\text{OH})_2\text{D}_3$ induced expression of E-cadherin, the expression of which has been linked to reduced metastatic potential of the cells (69). In an *in vivo* model of prostate cancer utilizing highly metastatic MAT LyLu and R3327-AT-2 Dunning PCa cells, $1\alpha,25(\text{OH})_2\text{D}_3$ treatment decreased the tumor size as well as the number and size of lung metastases (59).

Inhibition of Angiogenesis. Studies demonstrated the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit proliferation of endothelial cells *in vitro* (93-95), as well as reduce angiogenesis *in vivo* (96,97). In prostate cancer cell, $1\alpha,25(\text{OH})_2\text{D}_3$ interrupts interleukin 8 (IL-8) signaling, leading to inhibition of endothelial cell migration and tube formation (65). Interestingly, $1\alpha,25(\text{OH})_2\text{D}_3$ -treated tumor-derived endothelial cells (TDECs) induced apoptosis and cell cycle arrest, while endothelial cells isolated from normal tissues did not demonstrate such effects (93, 98). Chung et

al. demonstrated that TDECs can be more sensitive to $1\alpha,25(\text{OH})_2\text{D}_3$ due to epigenetic silencing of CYP24A1 gene (encoding $1\alpha,25(\text{OH})_2\text{D}_3$ -catabolizing enzyme 24-hydroxylase) (99).

Combinational studies. The efficacy of $1\alpha,25(\text{OH})_2\text{D}_3$ in PCa therapy seems to be dependent on the dose of $1\alpha,25(\text{OH})_2\text{D}_3$ administered. However, at higher concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$, the primary adverse side effect of $1\alpha,25(\text{OH})_2\text{D}_3$, hypercalcemia, becomes more prominent, limiting the maximum dose that can be given safely. Thus, several vitamin D analogs with high potency as antiproliferative agents and reduced hypercalcemic effects have been developed (reviewed in (100)). Another avenue to increase efficacy and decrease toxicity of $1\alpha,25(\text{OH})_2\text{D}_3$ is to use a combination of agents, at doses that are less than required when administered individually.

In vitro and *in vivo* analyses indicate that $1\alpha,25(\text{OH})_2\text{D}_3$ acts synergistically with multiple chemotherapeutic agents. $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to potentiate the anticancer activity of taxanes (101), platinum analogs (102-103), DNA-intercalating agents (104), ionizing radiation (105) and non-steroidal anti-inflammatory drugs (106).

In models of prostate cancer, $1\alpha,25(\text{OH})_2\text{D}_3$ significantly enhanced the growth inhibition induced by paclitaxel in PC3 cells *in vitro*, as well as in PC3-xenograph-bearing mice (101). The molecular basis for the enhanced anti-tumor activity of this combination was shown to be the increased expression of p21 by $1\alpha,25(\text{OH})_2\text{D}_3$, rendering the cells more sensitive to paclitaxel-induced apoptosis (101). Another study demonstrated synergistic inhibition of PCa cells by combining histone deacetylase inhibitors sodium butyrate and trichostatin with $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs (107). In this study, the mechanism appeared to involve neither p21 nor cell cycle arrest, but rather induction of apoptosis (107).

Synergistic growth inhibition by combination of $1\alpha,25(\text{OH})_2\text{D}_3$ and genistein was observed in several prostate cancer cell lines: in LNCaP cells, through cooperative up-regulation of VDR and p21 protein levels (108,109); in DU145, through genistein-mediated inhibition of enzymatic activity of CYP24, an enzyme involved in the catabolism of $1\alpha,25(\text{OH})_2\text{D}_3$ (110); and in PC3

cells, through inhibition of the prostaglandin pathway (111). The ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to regulate prostaglandin metabolism by repressing expression of prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2) was also implicated in the synergistic growth inhibition of prostate cancer cells by $1\alpha,25(\text{OH})_2\text{D}_3$ and nonsteroidal anti-inflammatory drugs (NSAIDs) (106).

Not-surprisingly, $1\alpha,25(\text{OH})_2\text{D}_3$ synergizes with azole antagonists of the primary vitamin D_3 catabolic enzyme CYP24A1 (112). One of them, ketoconazole, is being used for the treatment of androgen-independent prostate cancer (112).

In conclusion, the anticancer mechanisms of $1\alpha,25(\text{OH})_2\text{D}_3$'s action include induction of cell cycle arrest, inhibition of proliferation and angiogenesis, as well as inhibition of invasive and migratory potential of cancer cells. A large number of *in vivo* and *in vitro* studies support the antitumor effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs, and the potential for their effective use in combination with chemotherapeutic cancer agents.

1.3.3. Vitamin D use in Clinical Trials

The ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit growth of prostate cancer, as demonstrated in multiple *in vitro* and *in vivo* studies, led to the first vitamin D-based clinical trials in 1990s.

Trials Utilizing Vitamin D Compounds as a Single Agent. Use of $1\alpha,25(\text{OH})_2\text{D}_3$ as a single therapeutic agent for the treatment of prostate cancer was evaluated in small clinical trials, and some effectiveness was indicated by a slowing rate of PSA rise (113,114). Therefore, Osborn and colleagues tested $1\alpha,25(\text{OH})_2\text{D}_3$ in 13 hormone refractory metastatic prostate cancer patients in a phase II trial in 1995. In this study no objective response, determined as a >50% reduction in serum PSA levels, was observed (113).

Since this suggested that hormone refractory prostate cancer is too late of a stage to be treated with $1\alpha,25(\text{OH})_2\text{D}_3$, Gross et al. conducted a small study in which 7 men with early recurrent prostate cancer were treated with daily oral $1\alpha,25(\text{OH})_2\text{D}_3$ (0.5 – 2.5 $\mu\text{g/day}$) for 6-15 months (114). The rate of PSA rise during versus before $1\alpha,25(\text{OH})_2\text{D}_3$ therapy significantly decreased in

6 of 7 patients, while in the remaining man deceleration in the rate of PSA rise did not reach statistical significance. Hypercalciuria was the dose-limiting toxicity observed in all patients (114). Withdrawal from the therapy resulted in the resumption of the PSA rise, with doubling times returning to the values seen before the therapy. This suggests that $1\alpha,25(\text{OH})_2\text{D}_3$ has only cytostatic effect, slowing the progression of PCa as measured by PSA levels, without a reduction in tumor burden.

In an attempt to reduce the dose-limiting hypercalcemic toxicities, Beer et al. tested administration of $1\alpha,25(\text{OH})_2\text{D}_3$ at a very high oral dose using a once weekly regimen instead of daily administration as tested in the previous studies (115). Twenty-two prostate cancer patients with rising PSA after prostatectomy or radiation therapy received a weekly oral dose of $0.5 \mu\text{g/kg}$ $1\alpha,25(\text{OH})_2\text{D}_3$, which is approximately 70 times the physiologic replacement dose. Treatment was well tolerated, with no Grade ≥ 3 toxicity and no hypercalcemia or renal calculi. While no patient had a PSA response (determined as a 50% reduction in PSA level), three patients had confirmed reductions in PSA ranging from 10% to 47%. Statistically significant increases in the PSA doubling time (PSADT) were seen in three additional patients, and no patient had a shorter PSADT after starting treatment. For the entire study population, the median PSADT increased from 7.8 months to 10.3 months ($P = 0.03$)(115).

Another opportunity to reduce the hypercalcemic effects of administration of high doses of $1\alpha,25(\text{OH})_2\text{D}_3$ is to instead use $25(\text{OH})\text{D}_3$. Prostate cells express the 1α -hydroxylase enzyme, converting $25(\text{OH})\text{D}_3$ to $1\alpha,25(\text{OH})_2\text{D}_3$ locally (25, 44), and $25(\text{OH})\text{D}_3$ has been shown to inhibit growth of prostate cancer cells *in vitro* to a degree not significantly different from $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition (116,117). Taking this into account, Woo et al. studied 15 men with recurrent disease, and treated them with cholecalciferol ($25(\text{OH})\text{D}_3$) (2000 IU ($50\mu\text{g}$)) daily (118). PSA levels in 9 of 15 men decreased or remained unchanged for 21 months after the commencement of $25(\text{OH})\text{D}_3$. A statistically significant decrease in the rate of PSA rise after administration of $25(\text{OH})\text{D}_3$ ($P = 0.005$) compared with that before the treatment was observed.

The median PSADT increased from 14.3 months prior to commencing 25(OH)D₃ to 25 months after commencing 25(OH)D₃. Fourteen of 15 patients had a prolongation of PSADT, with no side-effects reported by patients (118). This study suggest a potential for the use of 25(OH)D₃ for the treatment of prostate cancer; however, it has to be taken into account that prostate cancer cells have been found to have a marked decrease in 1 α -hydroxylase activity compared to normal prostate cells (44,119,120).

Vitamin D Compounds in Combinational Regimens. Numerous phase I and phase II trials of 1 α ,25(OH)₂D₃ in combination with other therapies for treatment of prostate cancer showed the feasibility of administering 1 α ,25(OH)₂D₃ intermittently at high dose, a maneuver that lowers the calcemic effects of 1 α ,25(OH)₂D₃ (121-128). It has been demonstrated that the dosing technique is of particular importance in the systemic administration of 1 α ,25(OH)₂D₃ and its analogs, and that weekly dosing allowed substantial dose escalation without dose-limiting toxicities (129).

Beer et al. have studied high-dose oral 1 α ,25(OH)₂D₃ using a weekly schedule (2.6 μ g/kg weekly) with no dose-limiting toxicity (130). Later, using this weekly schedule, Beer et al. treated metastatic AIPC patients with docetaxel in combination with 1 α ,25(OH)₂D₃ and reported an 81% response rate for the combination versus an expected response of 40% to 50% for docetaxel alone (131).

In addition to their possible therapeutic effects in advanced prostate cancer, vitamin D metabolites might improve the quality of life in advanced prostate cancer patients. Beer and colleagues reported significant analgesic activity of 1 α ,25(OH)₂D₃ combined with docetaxel in men with metastatic AIPC (132). Another small study of men with metastatic AIPC treated with 2000 IU (international units) vitamin D for 12 weeks demonstrated there was an improvement in bone pain scores in 25% of patients and an improvement in muscle strength measurements in 37% of patients compared to placebo control (133).

Since $1\alpha,25(\text{OH})_2\text{D}_3$ use in clinics is primarily limited by side effects such as hypercalcaemia and hypercalcemia, several less calcemic analogs of calcitriol were tested in clinical trials for prostate cancer treatment and reviewed elsewhere (128).

Preclinical and early clinical data provide considerable rationale for continued research to evaluate use of vitamin D compounds and their analogs for the treatment of prostate cancer. However, before safe and efficient clinical use of $1\alpha,25(\text{OH})_2\text{D}_3$ and analogs is possible, well-designed trials will be needed to determine (i) the Maximum Tolerated Dose, (ii) an optimal phase II dose for use of vitamin D compounds in combination with cytotoxic agents, and (iii) optimal administration scheduling to allow for reduction of possible side-effects. Taken together, existing data support the potential for exploiting vitamin D compounds alone or in combination with other agents to control PCa progression, and further studies are warranted.

1.4. Role of PI3K/AKT Pathway in Prostate Cancer Development and Progression

1.4.1. PI3K/AKT Pathway

One of the central contributing factors in the survival of prostate cancer cells is the phosphoinositide-3 kinase PI3K-AKT pathway (134). Activation of PI3K can occur through tyrosine kinase growth factor receptors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R), cell adhesion molecules such as integrins, G-protein-coupled receptors, and oncogenes such as Ras. Following the activation of PI3K by tyrosine-kinase receptors or other cell surface receptors in response to ligands (e.g. insulin, PDGF, EGF, or FGF), PI3K catalyzes phosphorylation of the D3 position on phosphoinositides to generate the biologically active moieties phosphatidylinositol 3,4,5-triphosphate (PI-3,4,5-P3) and phosphatidylinositol 3,4-bisphosphate (PI-3,4-P2). Upon generation, PI-3,4,5-P3 binds to the pleckstrin homology (PH) domain of serine/threonine kinase AKT and 3'-phosphoinositide-

dependent kinase 1 (PDK1), recruiting them to the plasma membrane. This drives a conformational change in AKT, resulting in its phosphorylation by the constitutively active PDK1 at Threonine 308 (135,136) and by PDK2 (mammalian target of rapamycin complex 2 (mTORC2)) at Serine 473 (137). Inactivation of AKT through dephosphorylation is controlled by serine/threonine protein phosphatases PP1 and PP2A, with PP2A being the predominant AKT phosphatase (138,139).

Activated AKT translocates to the cytoplasm and nucleus, and activates downstream targets involved in survival, proliferation, cell cycle progression, growth, migration and angiogenesis. Phosphorylated AKT regulates cellular processes by phosphorylation of a number of substrates, including checkpoint kinase 1 (Chk1), murine double minute (MDM2), BclxL/Bcl-2 associated death promoter (BAD), the forkhead box O (FOXO) family of transcription factors, and tuberous sclerosis complex 2 (TSC2) (140). Another important substrate of AKTs is the mammalian target of rapamycin (mTOR), which plays a significant role in tumorigenesis (141).

AKT, also known as protein kinase B (PKB), is an evolutionarily conserved serine/threonine kinase. Three AKT isoforms (AKT1, AKT2 and AKT3), encoded by three separate genes, are expressed in mammalian cells. The three isoforms share >80% amino acid sequence identity and exhibit the same structural organization. AKT1 is the most ubiquitously expressed isoform in mammalian cells and tissues. AKT2 is also expressed in most tissues and organs, usually at lower levels than AKT1, except in insulin-responsive tissues, where it is expressed at a higher level (142,143). AKT3 is expressed at the lowest level in most adult tissues except testes and brain (144,145). While *Akt1*^{-/-} mice display mild growth retardation and increased apoptosis (146), the *Akt2*^{-/-} mice are insulin-resistant and display a diabetic phenotype (146,147), and the *Akt3*^{-/-} mice display a uniformly reduced brain size (148).

1.4.2. Implications of PI3K/AKT Pathway in Prostate Cancer

The PI3K/AKT pathway is greatly implicated in the development and progression of prostate cancer. Amplifications of PI3K have been reported (149); in addition, several protein-tyrosine kinases (PTKs) acting upstream of PI3K have been found to be overexpressed in prostate cancer. For example, *ErbB2/HER2/Neu* and IGF-1R were found to be elevated in some prostate cancers (150-152), while fibroblast growth factor receptor 1 (FGFR1) was shown to be overexpressed in localized cancer and amplified in hormone-resistant prostate cancer (149).

Downstream of PI3K, AKT is constitutively active in many cancers, including prostate cancer (149, 153). Levels of phospho-AKT are significantly higher in cancer cells relative to normal prostate epithelium and benign prostatic hyperplasia, further increasing in high-grade prostate tumors (154). Furthermore, increased levels of phospho-AKT were detected in AIPC tissues when compared with hormone-sensitive tissues, and were associated with decreased disease-specific survival (Hazard Ratio (HR) 2.89, Confidence Interval (CI) 1.43-5.8) (155). Overall, AKT expression and kinase activities have been shown to be associated with a poor prognosis (149,153), and phospho-AKT expression was found to be an independent predictor of biochemical recurrence (HR 3.44, CI 1.83-6.43) (156,157). Results of a study evaluating expression of AKT iso-forms with respect to prostate cancer recurrence showed that only high cytoplasmic AKT-1 combined with low nuclear AKT-1 independently predicted time to biochemical failure (HR 2.2; CI 1.12-3.99) (158).

AKT activation in prostate cancer can occur due to the activation of the PI3K pathway by gene amplification (149,153); but the most common reason for AKT activation is the loss of tumor suppressor phosphatase and a tensin homologue deleted in chromosome ten (PTEN) (159-163). The protein encoded by PTEN is a member of a family of dual-specificity phosphatases (164) which converts PI-3,4,5-P3 to PI-3,4-P2, preventing AKT recruitment to the plasma membrane and subsequent activation.

1.4.3. AKT Inhibition as Therapy

Recognizing AKT's central role in cancer development, multiple attempts have been made to identify or synthesize AKT inhibitors in academia, industry and government. To date, there are several classes of AKT inhibitors, including agents that target the pleckstrin homology domain or the ATP-binding pocket, allosteric inhibitors, pseudosubstrates, and isoform-selective AKT catalytic-domain inhibitors (165).

Several small-molecule compounds with inhibitory activities against AKT are being evaluated in early clinical trials, alone or in combination with other chemotherapeutics, including API-2 (Triciribin), GSK2141795, GSK2110183, SR13668, Ritonavir, Nelfinavir, Perifosine, and MK2206 (166,167). Several AKT inhibitors that are currently undergoing preclinical evaluation include PH-domain inhibitor PX-316 (ProlX Pharmaceuticals); highly selective AKT inhibitor A-443654 (Abbott Laboratories); and isoform-selective AKT inhibitors AKTi-1 and AKTi-2 (Merck, Inc), to name a few. In addition to AKT inhibitors, potent and isoform-selective PI3K inhibitors with improved pharmacologic properties (e.g. XL147 (Exelixis), BEZ235 (Novartis), and GDC-0941 (Genentech)) are now being developed, with some entering phase I clinical trials (140).

It is noteworthy that a number of chemopreventive compounds have been demonstrated to inhibit AKT. A few examples include curcumin (168), selenium (169), quercetin (170), genistein (171), apigenin (172) and silibinin (173). Most of the AKT inhibitory effects, however, have been shown *in vitro* and in some cases at doses far above those that are physiologically achievable.

Activation of AKT is a critical event in human cancer, and presents an attractive target for therapeutic inhibition. However, being one of the most physiologically relevant pathways, inhibition of the AKT pathway also presents a challenge in terms of possibly inducing severe toxicities. It is likely that AKT inhibitors will have to be used in combination with other chemotherapeutics to increase efficacy and reduce potential toxicities.

1.5. Senescence

Cell senescence, originally defined as the terminal state of cells with telomere dysfunction, is now understood to be a general reaction of cells to a wide range of cellular events (174-177). Cells that underwent senescence can not divide even if stimulated with mitogens, but they remain metabolically active and show characteristic changes in morphology, such as enlarged and flattened cell shape and increased granularity (Figure 4) (178).

1.5.1. Causes of cellular senescence

It is now apparent that many kinds of stimuli can induce a senescence response. Short and dysfunctional telomeres have been shown to trigger a senescence response (179,180) through triggering of a classical DNA-damage response (181,182). Severe DNA damage itself, occurring anywhere in the genome – especially damage that creates double-strand breaks (DSBs) – causes many cells to undergo senescence (183,184). Not surprisingly, many DNA damaging chemotherapeutic agents were shown to induce senescence of treated cells both in culture and *in vivo* (185). Senescence can also be triggered by chromatin perturbations (186,187). In addition, sustained signaling by certain anti-proliferative cytokines, such as interferon- β (188) or transforming growth factor- β (TGF- β) (189,190), as well as intracellular oxygen radicals, have been shown to induce cellular senescence (188). Last but not least, loss of a tumor suppressor or oncogene expression can lead to so-called oncogene-induced senescence. For example, overexpression of oncogenic H-Ras, K-Ras, and B-Raf (191-193), expression of constitutively active Akt1 (194), overexpression of Ctnnb1 (encoding β -catenin) (195), oncogenic activation of Myc (196), as well as loss of Pten (197) or Vhl (198) were shown to lead to senescence induction (these are reviewed in more detail in Chapter III). Results obtained in the studies on oncogene-induced senescence led to a suggestion that oncogene-induced senescence serves as a mechanism protecting oncogene-stimulated cells from malignant transformation (178,199,200).

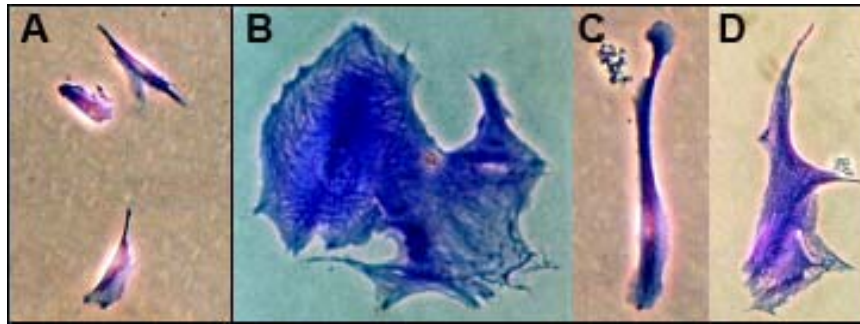


Figure 4. Morphology of senescent cells. Representative photographs of normal human fibroblasts (A) and fibroblasts showing senescent morphology (B,C,D). Senescent population has a more diverse phenotype (enlarged or elongated) than cells at earlier passages. [Copyright (2008) João Pedro de Magalhães, Integrative Genomics of Ageing Group. Reprinted with author's permission.]

1.5.2. Hallmarks of Senescence

One of the hallmarks of cellular senescence is an inability to progress through the cell cycle. Senescent cells arrest growth, usually with a DNA content that is typical of G1 phase, while remaining metabolically active (183, 201). Upon the cell cycle arrest, cells fail to initiate DNA replication despite adequate growth conditions. This replicative block is primarily caused by the expression of dominant cell-cycle inhibitors, with features and stringency dependent on species-, tissue- and cell-specific characteristics, as well as the genetic background of the cell. Most mouse fibroblasts senesce with a G1 DNA content (183). However, some oncogenes can cause a fraction of cells to senesce with a DNA content that is typical of G2 phase (202-204). In other systems, mutations in stress signaling pathways have been shown to induce G2-M arrest and senescence (205). Interestingly, another study showed that oncogene-driven DNA damage response led to senescence with cells stalling in S phase, which formed an augmented number of active replicons and exhibited defects of DNA replication fork progression (202). Moreover, it appears that at least a subset of oncogenes can only trigger senescence if cells are allowed to enter S phase (202, 206).

The best-studied cellular system of senescence is the senescence of normal fibroblasts. In these cells senescence often is initiated with activation of p53. In the case of replicative senescence, p53 protein is stabilized through the involvement of p14^{ARF}, a tumor suppressor that sequesters the murine double minute 2 (MDM2) protein (207) or promyelocytic leukemia (PML) tumor suppressor, which regulates p53 acetylation (208,209). One of the most relevant events following p53 activation is transcriptional activation of p21^{Waf1/Cip1} (210) which leads to induction of cell cycle arrest in senescent cells (211,212). Upon establishment of growth arrest, the activation of p53 and p21 in senescent cells decreases while another CDK inhibitor, p16^{Ink4A}, becomes constitutively up-regulated, suggesting that p16 might be responsible for growth arrest maintenance in senescent cells (212,213) (Figure 5). Thus, the two cell cycle inhibitors most commonly expressed in the senescent cells are the CDKIs p21 and p16 (178), which are critical

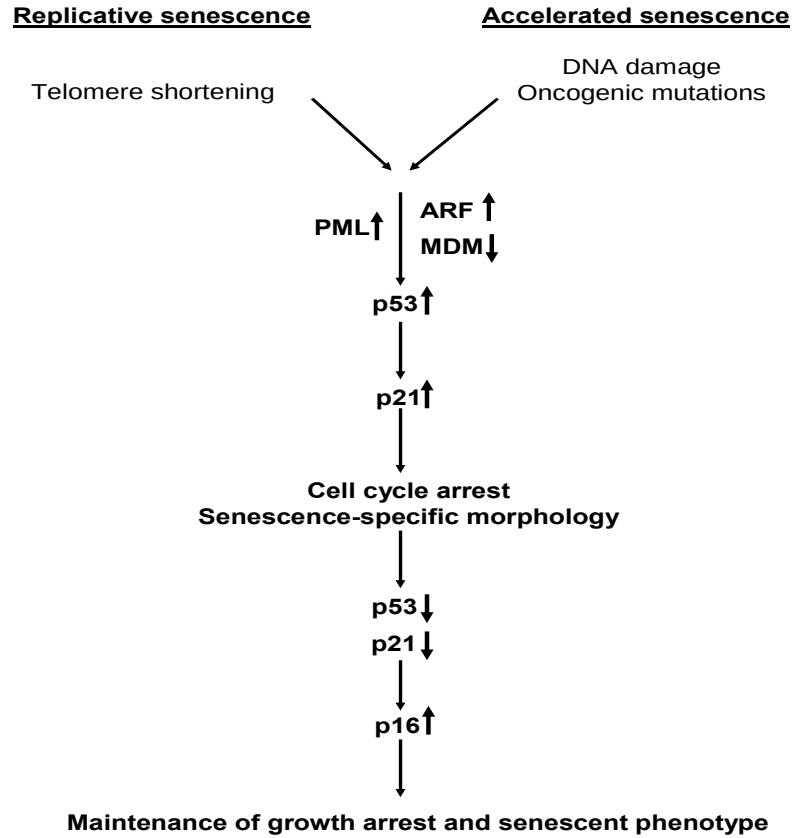


Figure 5. Key events in the induction of senescence of normal fibroblasts. ARF, alternate reading frame; MDM, murine double minute; PML, promyelocytic leukemia

components of tumor-suppressor pathways governed by the p53 and pRb proteins, respectively. Both, p21 and p16 ultimately maintain pRb in a hypophosphorylated and active state. Other inhibitors, such as p27^{Kip1} (200) and p15^{Ink4b} (214) were also shown to play a role in some instances.

1.5.3. Markers of senescence

The most widely used surrogate marker of senescent cells is the senescence-associated β -galactosidase activity (SA- β -gal), which is detectable by X-gal staining at pH 6.0 (215). SA- β -gal appears to reflect increased lysosomal mass of senescent cells (216). Other commonly used markers of senescence are the same proteins involved in the mechanism of cell growth arrest including the products of CDKN2A locus (INK4A and ARF) (217), as well as cell cycle regulators CDKIs p21 and p27 (194, 198). p16, an important regulator of senescence, is now used to identify senescent cells (218). However, p16 is expressed by many, but not all, senescent cells (219), and it is also often expressed in tumors that have lost pRb function (220). More recently, molecules involved in the DNA damage response (such as γ H2AX) or the formation of senescence-associated heterochromatin foci (SAHF) (such as heterochromatin protein gamma (HP1 γ)) have been used as surrogate markers of the senescence process (182,202,221,222). However, these molecules are not exclusive to senescence or are not fully characterized. Recently, senescence markers were proposed to be used as diagnostic and prognostic tools (223). Despite the growing interest in defining *in vitro* and *in vivo* cellular senescence and the efforts of many laboratories, there are still only a few robust markers of senescence (223).

1.5.4. Senescence induction in cancer therapy

For a long time it was a common assumption that neoplastically transformed cells are no longer capable of inducing senescence. Today it is known that tumor cells can undergo senescence, and can be forced into this process by various stimuli including genetic

manipulations, epigenetic factors, conventional anticancer agents, radiation and differentiation agents (reviewed in (185)).

Many chemotherapeutic drugs cause severe DNA damage and induce senescence. Since many cancers have at least in part lost the ability to induce apoptosis, senescence-inducing drugs could represent a potential alternative approach to treat tumors that are resistant to apoptosis-based therapies. It is not well-understood what determines whether a cell will undergo apoptosis or senescence in response to treatment. It could depend on the dose of the chemotherapeutic agent, with low doses inducing senescence and with high doses inducing apoptosis (224,225). In addition, defects that have accumulated in a cancer cell are strong determinants of a possible treatment outcome. The same chemotherapeutic treatment was shown to induce either apoptosis or senescence, depending on the genetic alterations present in tumors in mice (226).

Drug-induced senescence has been observed in *in vitro* models by a variety of biochemically unrelated DNA-damaging anticancer agents, such as the topoisomerase I inhibitor camptothecin, the topoisomerase II inhibitor adriamycin, the cross-linking agent cisplatin, γ -irradiation and the anti-metabolite cytarabin, while anti-microtubule agents did not seem to induce significant senescence (227).

Two recent reports analyzing senescence markers in biopsies from patients with lung or breast cancer after neoadjuvant chemotherapy have observed chemotherapy-induced senescence, which was associated with treatment success (228,229). te Poele and colleagues demonstrated that 50% of tumor samples from breast cancer patients receiving neoadjuvant therapy stained positive for SA- β -gal, demonstrated high-levels of p16/INK4a and p53 expression, and displayed senescence-like growth arrest (229). In contrast, normal tissue from adjacent areas was completely SA- β -gal negative, demonstrating a high specificity in senescence induction of the cancer tissue. In addition, about 10% of cancer cells were SA- β -gal positive before any treatment was applied, suggesting the occurrence of 'spontaneous' senescence.

Thus, there is some evidence that chemotherapeutic drugs can trigger senescence of cancer cells in human tumors, and it is possible that senescence could contribute to the success of chemotherapy (228,229). However, more *in vivo* studies on the long-term impact of the induction of senescence are needed.

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CHAPTER II

1 α ,25-DIHYDROXY VITAMIN D₃ AND PI3K/AKT INHIBITORS SYNERGISTICALLY INHIBIT GROWTH AND INDUCE SENESCENCE IN PROSTATE CANCER CELLS

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2.1. ABSTRACT

BACKGROUND: 1- α , 25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) inhibits proliferation of multiple cancer cell types including prostate cells, and upregulates p21 and/or p27, while loss of Pten and PI3K/AKT activation stimulates cancer cell survival, and downregulates p21 and p27. We hypothesized that inhibition of the PI3K/AKT pathway would synergize with the antiproliferative signaling of 1 α ,25(OH)₂D₃. **METHODS:** Viability, cell cycle and senescence of cells were evaluated upon combinational treatment with 1 α ,25(OH)₂D₃ and pharmacological PI3K/AKT inhibitors. **RESULTS:** Pharmacological inhibitors of PI3K or AKT and 1 α ,25(OH)₂D₃ synergistically inhibited growth of DU145 and LNCaP prostate cancer cell lines, and also of primary human prostate cancer cell strains. One of the inhibitors used in the study, API-2 (Triciribine), is currently in clinical trials for treatment of cancer. A novel mechanism for the antiproliferative effects of 1 α ,25(OH)₂D₃ in prostate cells, induction of senescence, was discovered. Combination of 1 α ,25(OH)₂D₃ and AKT inhibitor cooperated to induce G₁ arrest, senescence, and higher p21 levels in prostate cancer cells. **CONCLUSIONS:** These findings provide a rationale for the development of therapies utilizing 1 α ,25(OH)₂D₃ or its analogs combined with inhibitors of PI3K/AKT for the treatment of prostate cancer.

2.2. INTRODUCTION

2.2.1. Prostate Cancer

Prostate cancer (PCa) is the second most commonly diagnosed cancer and the second most common cause of cancer death in American men (1). Androgen ablation is the first-line of treatment for advanced prostate cancer, and leads to significant apoptosis of prostatic cancer cells. However, within 18-24 months after androgen ablation, the majority of patients relapse with androgen-independent prostate cancer (AIPC). The median survival once AIPC develops is 12 to 18 months. Thus, therapies that can prevent PCa, prolong the hormone-dependent state, or are effective against androgen-independent prostate cancer are urgently needed.

2.2.2. Role of Vitamin D in Prostate Cancer

Risk factors associated with PCa include age, race, as well as genetic, dietary, and hormonal influences (2,3). Vitamin D deficiency has also been suggested as a factor contributing to the risk of prostate cancer development (4,5). Vitamin D is a hormone that can be obtained from the diet and is produced endogenously, and is then transformed by a series of reactions that culminate in the most active metabolite of vitamin D, $1\alpha, 25(\text{OH})_2\text{-vitamin D}_3$ ($1\alpha, 25(\text{OH})_2\text{D}_3$). $1\alpha, 25(\text{OH})_2\text{D}_3$ elicits antiproliferative effects in a variety of cancer cell types, including cell lines derived from prostate (6,7). The ability of $1\alpha, 25(\text{OH})_2\text{D}_3$ to inhibit prostate cell growth has also been demonstrated in primary prostatic cells from histologically normal benign prostatic hyperplasia, prostate cancer specimens (8), multiple prostate cancer cell lines (9-11), xenograft models of prostate cancer (12,13) and in the Dunning rat prostate model (14). The anticancer mechanisms of $1\alpha, 25(\text{OH})_2\text{D}_3$'s action include induction of cell cycle arrest, promotion of differentiation, inhibition of proliferation and angiogenesis, as well as inhibition of the invasive and migratory potential of cancer cells (reviewed in (15) and in Chapter I).

Classical actions of $1\alpha,25(\text{OH})_2\text{D}_3$ are mediated through the vitamin D receptor (VDR), which is a member of a superfamily of nuclear steroid hormone receptors. Upon $1\alpha,25(\text{OH})_2\text{D}_3$ binding, VDR translocates to the nucleus, dimerizes with retinoid X receptor (RXR) and modulates the expression of target genes. Although a number of $1\alpha,25(\text{OH})_2\text{D}_3$ responsive genes are known, the exact mechanism of growth regulation by $1\alpha,25(\text{OH})_2\text{D}_3$ is not completely defined; however, an increase in p21 and/or p27 is an almost universal feature (6).

2.2.3. Implications of PI3K/AKT Pathway in Cancer

One of the central contributing factors in the survival of cancer cells is activation of the phosphoinositol-3 kinase PI3K/AKT pathway. Activated AKT phosphorylates a host of proteins that affect cell growth, cell cycle entry and cell survival. AKT generally acts to promote survival through inhibition of pro-apoptotic factors and activation of anti-apoptotic factors. For example, AKT phosphorylates multiple members of Forkhead family of apoptotic regulators, causing their translocation from the nucleus to the cytoplasm and thus inhibiting the transcription of proapoptotic genes (16). AKT also inhibits proapoptotic factors such as BAD, BAX and BID, while activating anti-apoptotic factors such as Bcl-xL (17-19). In addition, AKT is involved in regulation of oncoprotein MDM2 resulting in inactivation of p53 (20,21). Furthermore, activation of the mammalian target of rapamycin (mTOR) by AKT can also lead to increased proliferation and induction of pro-angiogenic gene expression (22). Regulation of the cell cycle is also affected by AKT signaling, with primary targets including cyclin D, p21 and p27, and many others (22).

AKT activation is commonly implicated in PCa (23) and most frequently occurs through silencing of PTEN (24). Up-stream of AKT, PI3K and various growth factor receptors (e.g. receptors for FGF, EGF and IGF) are overexpressed in some PCa, leading to increased AKT signaling (22). Regardless of the molecular mechanisms responsible, excessive activation of AKT is a poor prognostic marker, predicting poor survival and a high risk of biochemical recurrence following radical prostatectomy (25).

2.2.4. Interaction between AKT/PTEN Axis and $1\alpha,25(\text{OH})_2\text{D}_3$ Signaling: Role of p21, p27

Existing data demonstrate that loss of PTEN and activation of AKT downregulate the expression of p21 and p27 by a number of mechanisms (26-30). On the other hand, the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs almost universally involve upregulation of p21 and/or p27 (Figure 1) (6).

$1\alpha,25(\text{OH})_2\text{D}_3$ can directly upregulate expression of p21 through the vitamin D response elements (VDRE) in its promoter (31). Unlike p21, p27 does not have a VDRE in its promoter, but it can be transcriptionally upregulated by $1\alpha,25(\text{OH})_2\text{D}_3$ through increased binding of the Sp1 transcriptional factor to the Sp-1 binding site within the promoter of p27 (32). Degradation of p27 and p21 is regulated by the activity of the SCF-Skp2 protein complex, which targets phosphorylated proteins for ubiquitin-dependent proteolysis (33). $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs were shown to increase levels of both p21 and p27 through downregulation of Skp2 (34,35), and also through inhibition of CKS1, which is another member of the Skp2 complex playing a role in degradation of p27 (35,36).

Absence of PTEN and subsequent activation of AKT, on the other hand, have been shown to negatively regulate p27 and p21 levels, which can occur through several mechanisms. First, activated AKT can directly phosphorylate and inhibit forkhead transcription factors which induce transcription of p27 (27, 40-43). Second, active AKT can directly phosphorylate p27 and p21, leading to their cytoplasmic retention and degradation (26, 46). Third, dephosphorylation of p27 by PTEN has been demonstrated to reduce association between p45^{Skp2} and p27^{Kip1} (34), which leads to decreased degradation of p27. Fourth, PTEN positively regulates levels and activity of p53, which is a transcriptional regulator of p21 and p27 levels (38,39). Conversely, activated AKT leads to increased activity of MDM2, which then promotes p53 degradation (20,21). Moreover, in clinical PCa, loss of PTEN expression was associated with reduced expression of p27 (47) and increased expression of Skp2 (48).

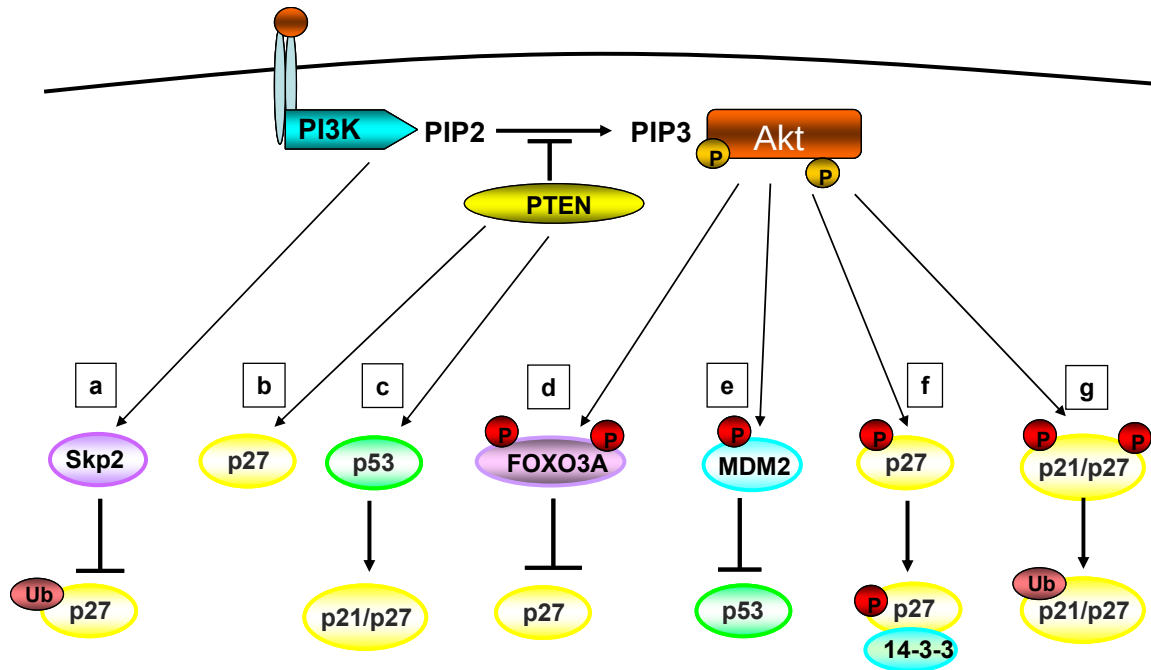


Figure 1. Interactions of PTEN/AKT Axis and p21/p27.

While the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ almost universally involve upregulation of p21 and p27 (3,4), loss of PTEN and activation of AKT can negatively regulate p27 and p21 levels through several mechanisms: **(a)** PI3K signaling can upregulate protein levels of SKP2 leading to increased degradation of p27 (37); **(b)** dephosphorylation of p27 by PTEN can reduce association between p45Skp2 and p27 leading to decreased degradation of p27 (34); **(c)** PTEN can positively regulate levels and activity of p53, which is a transcriptional regulator of p21 and p27 levels (38,39); **(d)** activated AKT can directly phosphorylate and inhibit forkhead transcription factors which induce transcription of p27 (27, 40-43); **(e)** activated AKT can increase activity of MDM2, which then promotes p53 degradation (20,21); **(f)** Akt-dependent phosphorylation of p27 can promote binding to 14-3-3 and cytoplasmic localization (44,45); **(g)** activated AKT can directly phosphorylate p27 and p21, leading to their cytoplasmic retention and degradation (26, 46).

p21 and p27 have to be present in the nucleus in order to function as CDK inhibitors. Recent findings, however, demonstrate that p27 can localized to the cytoplasm (44, 49-52), and when sequestered to the cytoplasm, p27 may play an anti-apoptotic role (53,54). This is comparable to p21, which also migrates to the cytoplasm (55), leading to an increased resistance to apoptosis (56). It can be speculated that the ability of AKT to phosphorylate p21 and p27, leading to their cytoplasmic retention (26, 46), can contribute to AKT-mediated apoptosis resistance.

Taken together, these data indicate that lost PTEN and subsequent activation of AKT can lead to lower levels of p27 and p21, which are the primary targets of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition.

2.2.5. Inhibition of AKT Pathway as a Cancer Therapeutic

The AKT pathway presents an attractive target for anticancer therapies, and several AKT inhibitors with strong anticancer activity in preclinical and clinical studies have been developed (57). Several classes of AKT inhibitors include isoforms-specific inhibitors, pseudosubstrates, agents that target the pleckstrin homology domain or the ATP-binding pocket and others (58).

Although activation of AKT is a commonly observed and critical event in the progression of human cancer and presents an appealing target for therapeutic inhibition, the AKT pathway is a highly physiologically relevant pathway (59). As a result, its inhibition is expected to induce severe toxicities. It is thus likely that AKT inhibitors will have to be used in combination with other chemotherapeutics, in order to increase the efficacy and reduce potential toxicities.

In the present study we evaluated the effects of combination treatments, comprised of $1\alpha,25(\text{OH})_2\text{D}_3$ plus a PI3K/AKT inhibitor, on the growth of prostate cancer cells. We used several inhibitors of PI3K and/or AKT, including API-2 and GSK690693, which have been tested for treatment of malignancy in clinical trials.

API-2 (Triciribine) was identified in 1970 by screening NCI's chemical libraries, and was found to be a tricyclic nucleoside that inhibits the pleckstrin homology (PH) domain of AKT

(Figure 2) (60). API-2 inhibits AKT kinase activity and stimulates apoptosis in xenographs of human breast, prostate, ovarian and pancreatic cancer cells exhibiting high AKT activity (61,62). API-2 is highly selective for AKT, and does not inhibit the activation of PI3K, PDK1, protein kinase C or protein kinase A (62). Interestingly, API-2 was tested for its antitumor activity in phase I and phase II trials conducted over 20 years ago (61,63,64). Due to excessive toxicities (e.g. hyperglycemia, hepatotoxicity and thrombocytopenia), the drug was not further considered as a treatment option. Nowadays, API-2 (Triciribine) has been of increasing interest for its potential use in treating cancer, and this has led to currently on-going phase I trials that are evaluating use of Triciribine Phosphate Monohydrate (TCN-PM, VD-0002) for the treatment of advanced hematologic malignancies (ClinicalTrial.gov identifier NCT00642031) and metastatic cancer (ClinicalTrial.gov identifier NCT00363454).

Another AKT inhibitor, GSK690693, synthesized at GlaxoSmithKline, is an ATP-competitive kinase inhibitor (65) (Figure 3). GSK690693 inhibits AKT in a time-dependent and reversible manner ($t_{1/2}$ for Akt1 and Akt2 of 38min and 30min), and has IC_{50} values of 2, 13, and 9 nmol/L for Akt1, 2, and 3, respectively (65). Daily administration of GSK690693 was shown to produce significant antitumor activity in mice bearing various xenografts, including LNCaP xenografts (65). In 2007, GSK690693 entered phase I clinical trial, which recently was terminated (ClinicalTrial.gov identifier NCT00493818).

Based on the previous evidence demonstrating that the antiproliferative effects of $1\alpha,25(OH)_2D_3$ involve upregulation of p21 and/or p27, while activation of PI3K/AKT downregulates their expression, we hypothesized that pharmacological inhibitors of AKT will cooperate with the antiproliferative actions of $1\alpha,25(OH)_2D_3$ in prostate cancer cells. Our results demonstrated that combination of $1\alpha,25(OH)_2D_3$ and pharmacological inhibitors of AKT synergize to inhibit growth of prostate cancer cells and cooperate to induce G1 arrest, senescence, and higher p21 protein levels. These findings provide a rationale for the development of

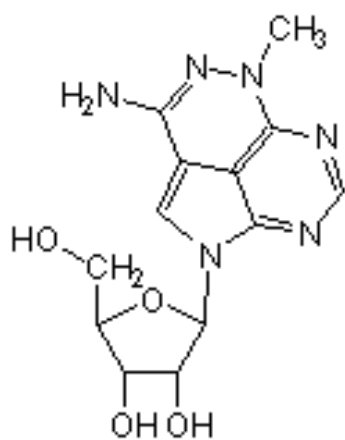
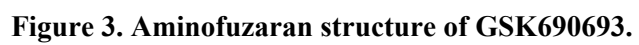


Figure 2. Structure of tricyclic nucleoside API-2.



the improved therapies utilizing $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs combined with inhibitors of PI3K/AKT for the treatment of prostate cancer.

2.3. MATERIALS AND METHODS

Materials. $1\alpha,25(\text{OH})_2\text{D}_3$ (Biomol, Plymouth Meeting, PA) was reconstituted in 100% ethanol and stored at -80°C . LY294002 (Sigma-Aldrich Co., St Louis, MO). GSK690693 (65) (a generous gift from GlaxoSmithKline, Collegeville, PA), Rapamycin (Calbiochem, La Jolla, CA) (a generous gift from Dr. Steven Kridel (WFU)) and API-2 (62) (Calbiochem, La Jolla, CA) were reconstituted in DMSO and stored at -20°C .

Tissue Culture. LNCaP and DU145 cells (both from American Type Culture Collection, Manassas, VA) were grown in RPMI-1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin. Human prostate epithelial cancer cell strain WFU273Ca was isolated from fresh human prostate (prostate cancer, Gleason grade 6), validated for histological origin and maintained as previously described (66). Acquisition of the human specimen from radical prostatectomies was performed at Wake Forest University School of Medicine in compliance with Institutional Research Board requirements. Briefly, a small piece of tissue was removed and minced. The tissue was then digested with collagenase overnight. To remove the collagenase and the majority of the stromal cells, the tissue was rinsed and centrifuged. The tissue was inoculated into a tissue culture dish coated with collagen type I (Collagen Corporation, Palo Alto, CA), and was grown in medium PFMR-4A (67) supplemented with growth factors and hormones as described (66). The histology of each specimen was verified by inking and fixing the prostate tissue after dissection and serially sectioning the marked area, as well as the sections immediately adjacent to the area of the dissection. The cells that grew out from the tissue were aliquoted and

stored in liquid nitrogen. The frozen aliquots were thawed to produce secondary cultures, which were grown in medium MCDB 105 (Sigma, St. Louis, MO) supplemented with growth factors and hormones as described (66).

Growth Assays for Synergism Determination. Cells were plated at 10^4 cells per 35 mm dish in triplicate. To determine synergism, cells were treated with increasing doses of AKT inhibitor, increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$ or multiple combinations of AKT inhibitor and $1,25(\text{OH})_2\text{D}_3$. Briefly, 48 hours after plating, the cell growth medium was replaced with 1 ml of experimental medium containing twice (2x) the indicated concentration of a PI3K/AKT inhibitor or vehicle (DMSO, 1x = 0.1% V/V). One hour later, 1 ml of medium containing twice the final concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ or vehicle (ethanol, 1x = 0.1% V/V) was added to each dish. An AKT inhibitor was applied 1 hour prior to the $1\alpha,25(\text{OH})_2\text{D}_3$ treatment, as API-2 was shown to reduce phosphorylation of AKT and phosphorylation of AKT's downstream targets (Bad, AFX, and GSK-3 β) as early as at 1 hour post treatment (62). In addition, our preliminary tests demonstrated that application of AKT inhibitor 1 hour prior to $1,25(\text{OH})_2\text{D}_3$ treatment demonstrated moderately stronger synergism of growth inhibition compared to 0 hours, 4 hours, 8 hours and 24 hours between the treatments (data not shown). DU145 and LNCaP cells remained in the experimental medium until the vehicle control cells reached 80-90% confluence, typically 5-7 days. For WFU273Ca and for all experiments using GSK690693, the experimental medium was replaced every 48 hours. Viable cells were counted with a hemacytometer after trypan blue exclusion. Results of representative experiments are shown.

Flow Cytometry. Flow cytometry was performed as described (7) using a Becton Dickinson FACSCaliber, and results were analyzed by the Cell Quest Pro v.6.0 program (Becton Dickinson, Mansfield, MA). Data were processed with ModFit LT v.2.0 software (Verity Software House,

Topsham, MN). Each treatment was performed in triplicate for each experiment, and each experiment was conducted three times.

Senescence-associated SA- β -galactosidase (SA- β -gal) Activity. Cells were cultured and treated as described above. After 5-7 days of treatment, SA- β -galactosidase activity was evaluated by the method of Dimri et al. (68). For positive controls, SA- β -gal activity was evaluated in cells treated with 100 nM Doxorubicin; for negative controls, SA- β -gal activity was evaluated in cells treated with 100 nM Doxorubicin at pH 7, which inhibits SA- β -gal activity. Digital images were taken from 10 random areas at 20X magnification. Digital images were evaluated in Photoshop CS2 9.0.2 (Adobe Systems, San Jose, CA). The number of SA- β -gal positive cells was counted in each image and presented as percent of total cell number $\pm$ SE.

Immunoblot. Cells were collected from monolayer by light trypsinization, and cell pellets were resuspended in lysis buffer (20 mM HEPES, 10 mM NaCl, 3 mM MgCl₂, 0.1% NP40, 10% glycerol, 0.2 mM EDTA) containing freshly added 1 mM DTT and 0.4 mM PMSF. Tubes with cells were left on ice for 15min. 50 μ g of protein from each tube was subjected to electrophoresis on SDS-PAGE gels. Proteins were transferred onto prewetted Hybond-P PVDF (polyvinylidene difluoride) membranes according to the manufacturer's protocol (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were incubated with appropriate dilutions of Pten (sc-7974) (Santa Cruz Biotechnology, Santa Cruz, CA), p21 (556430) and p27 (610241) antibodies (BD Pharmingen, San Diego, CA). Secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnology) were used. The ECL Plus Western Blotting Detection System kit (Amersham-Pharmacia) was used for detection of proteins. Images were analyzed by ImageQuant TL v. 7.0 (GE Healthcare, Piscataway, NJ).

Statistical Analyses. Synergism was assessed with CalcuSyn software (Biosoft, Ferguson, MO) as previously described (7). Briefly, the dose effect for each drug alone was determined based on the experimental observations using the median effect principle: the combination index (CI) for each combination was calculated according to the following equation: $CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [(D)_1(D)_2/(D_x)_1(D_x)_2]$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination, and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. $CI = 1$ represents the conservation isobologram and indicates additive effects. CI values < 1 indicate a more than expected additive effect (synergism). The Dose Reduction Index (DRI) for each drug at each dose was calculated using the equation: $(DRI)_1 = (D_x)_1/D_1$ and $(DRI)_2 = (D_x)_2/D_2$. Statistical analyses for synergism experiments were performed using the statistical software package NCSS 2002 (Number Cruncher Statistical Systems, Kaysville, UT). Differences in growth data were determined by two-way ANOVA, controlling for $1\alpha,25(OH)_2D_3$ or PI3K/AKT inhibitor dose with post hoc analysis using the Fisher's test. Cell cycle distribution and senescence analyses were performed using two-way ANOVA, with post hoc analysis using the Fisher's LSD test. In all cases, $P \leq 0.05$ was considered significant. Data were analyzed in PROC MIXED in SAS v9.2.

2.4. RESULTS

2.4.1. $1,25(OH)_2D_3$ and PI3K/AKT Inhibitors Synergistically Inhibit Growth of

Prostate Cancer Cells

To test whether inhibition of the PI3K/AKT pathway synergizes with $1\alpha,25(OH)_2D_3$ treatment to inhibit the growth of prostatic cells, we first utilized a pharmacological inhibitor of PI3K/AKT, LY294002 (69). Cells were treated with increasing doses of $1\alpha,25(OH)_2D_3$ or LY294002 or with multiple combinations of the two compounds, and the number of viable cells

was assessed. This was followed by assessment of synergism using the Chou-Talalay method (70) in which the combination index (CI) values < 1 indicates presence of synergism.

We found that LNCaP cells, which lack functional PTEN and are moderately sensitive to growth inhibition by $1\alpha,25(\text{OH})_2\text{D}_3$, demonstrated statistically significant synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ and LY294002 at lower doses of $1\alpha,25(\text{OH})_2\text{D}_3$ (Figure 4A, Table I). The combination of 1 nM $1\alpha,25(\text{OH})_2\text{D}_3$ treatment with 0.2 μM , 1 μM or 5 μM LY294002 demonstrated statistically significant synergism as determined by CI values from isobologram analysis (Table I). The dose-reduction index (DRI) for each of the drugs is also depicted in Table I, and presents a predictive measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone (70).

DU145 cells (wild-type PTEN) are known to be resistant to the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ (71). Consistent with this, we found that $1\alpha,25(\text{OH})_2\text{D}_3$ did not significantly affect growth of DU145 cells (Figure 4B). While 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ did not inhibit growth of DU145 cells, pretreatment of DU145 cells with 5 μM LY294002 sensitized the cells to $1\alpha,25(\text{OH})_2\text{D}_3$ treatment, leading to a reduction in the number of viable cells to 40% compared to treatment with LY294002 alone (Figure 4B). Significant synergism was observed across multiple doses of both compounds (Table I).

As LY294002 has recently been reported to have many other off-target effects (72), we sought to validate synergism with AKT-inhibition specifically. To do so, we used two different AKT inhibitors: API-2 (Triciribine), which selectively inhibits Akt1/2/3 without inhibiting PI3K or PDK (62), and GSK690693, an ATP competitive AKT inhibitor (65).

The combination of $1\alpha,25(\text{OH})_2\text{D}_3$ with API-2 elicited synergistic growth inhibition of DU145 cells (Figure 5A, Table II). DU145 cells treated with the combination of API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ demonstrated strong synergism at all doses tested, as determined by CI values from isobolograms (Table II). Note that the treatment with API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ was applied

only once, after which the cells were allowed to grow until the cells in either of the treatment groups were 80-90% confluent.

Next, we tested for the presence of synergism in primary human prostate cancer epithelial cells. Human primary epithelial cell strain WFU273Ca was isolated from a fresh human prostate with histologically confirmed prostate cancer of Gleason grade 6. The strain was shown to be PTEN-positive by immunoblot, while still demonstrating high levels of activated phosphorylated AKT (Figure 5B, insert). The combination of API-2 and $1,25(\text{OH})_2\text{D}_3$ applied to the WFU273Ca strain inhibited cell growth demonstrating statistically significant synergism between the compounds (Figure 5B, Table II), with DRI values as high as 15.3 for $1,25(\text{OH})_2\text{D}_3$ and 14.6 for API-2.

Even though both the WFU273Ca cell strain and the DU145 cell line, are PTEN-positive, WFU273Ca shows activation of the AKT pathway, as demonstrated by phospho-AKT levels (Figure 5B, insert), while DU145 consistently demonstrated undetectable levels of pAKT (data not shown). The mechanistic basis for activation of AKT in WFU273Ca is not known. The higher sensitivity of the WFU273Ca cell line to growth inhibition by AKT inhibitor API-2 might be explained by a possible dependency of WFU273Ca on AKT activation for proliferation of the cells. The combination of GSK690693 with $1,25(\text{OH})_2\text{D}_3$ also elicited strong to very strong synergistic growth inhibition in DU145 cells (Figure 5C, Table II), as well as moderate to very strong synergism in WFU273Ca human primary prostatic epithelial cells (Figure 5D, Table II). The mammalian target of rapamycin complex 2 (mTORC2), which functions downstream of AKT, has been a promising target for anticancer therapeutics (73). Rapamycin and its analogs are inhibitors of mTORC2 that are currently being tested in clinical trials (73). In view of this, we also evaluated the effect of the combination of Rapamycin and $1,25(\text{OH})_2\text{D}_3$ on growth of DU145. No cooperation between the two drugs was observed (Figure 6).

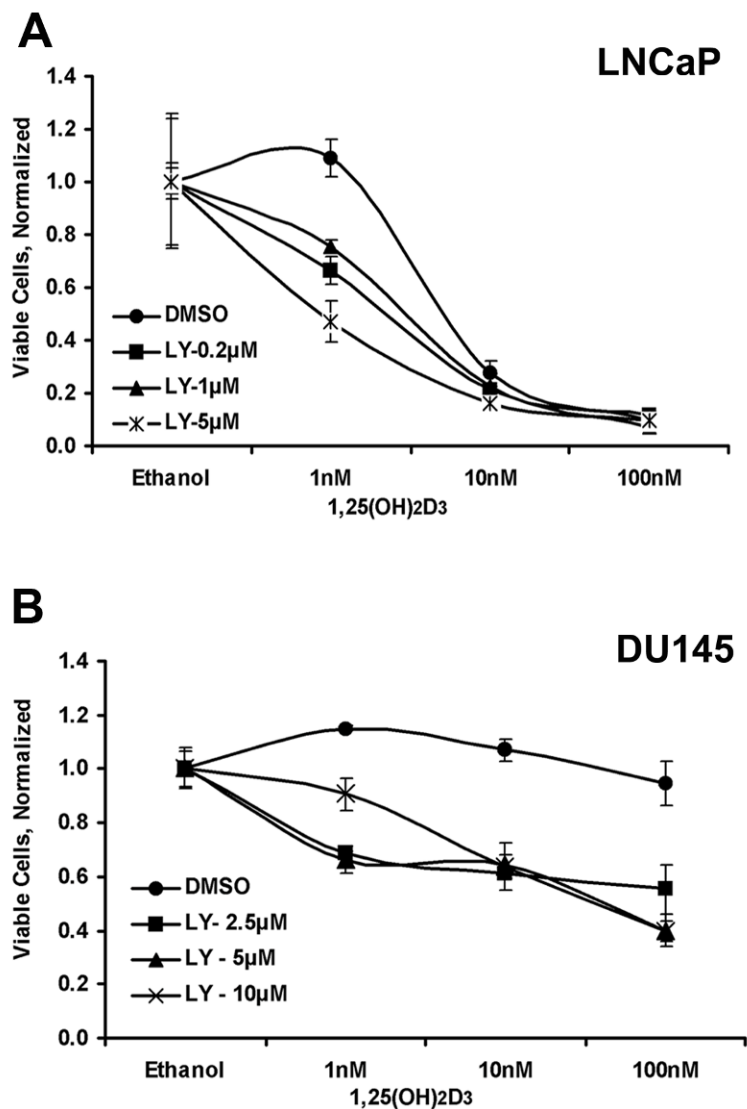


Figure 4. LY294002 and 1 α ,25(OH)₂D₃ synergistically inhibit growth of LNCaP and DU145 cells. Growth inhibition of LNCaP(A) and DU145(B) in response to LY294002 and 1 α ,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in Materials and Methods. Each point represents the mean and standard deviation of triplicate plates after normalization of cell number to controls (0.1% ethanol).

TABLE I

Synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ and LY294002 in LNCaP and DU145 cells***

	1,25(OH) ₂ D ₃ , nM	LY294002, μM	CI*	DRI**		Synergism
				1,25(OH) ₂ D ₃	LY294002	
LNCaP						
	1	0.2	0.171	8.2	20.7	Strong
	1	1	0.298	10.9	4.8	Strong
	1	0.5	0.675	26.3	1.6	Moderate
DU145						
	1	2.5	0.408	513.8	2.5	Intermediate
	1	5	0.650	666.0	1.5	Intermediate
	10	2.5	0.382	58.2	2.8	Intermediate
	10	5	0.654	67.7	1.6	Intermediate
	10	10	0.801	117.6	1.3	Moderate
	100	2.5	0.489	6.4	3.0	Intermediate
	100	5	0.585	9.5	2.1	Intermediate
	100	10	0.701	14.9	1.6	Intermediate

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism).

**Dose-reduction Index. DRI is calculated using the equation: $(\text{DRI})_1 = (D_x)_1 / D_1$ and $(\text{DRI})_2 = (D_x)_2 / D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination, and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone.

*** Data are from assays depicted in Figure 4; only mean values that are statistically significantly different by ANOVA are depicted.

The observed synergism between AKT inhibitors and $1\alpha,25(\text{OH})_2\text{D}_3$ is not limited to prostate cancer cells, as pancreatic cancer cell lines Panc-1 (Appendix, Figure AP-1, Table AP-I) and BXPC-3 (Appendix, Figure AP-2, Table AP-II) also demonstrated ‘moderate’ to ‘very strong’ synergism between AKT inhibitor API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$. It is noteworthy that pancreatic cancer cell lines were less sensitive to AKT inhibition and required higher doses of API-2 compared to prostate cancer cell lines.

2.4.2. $1\alpha,25(\text{OH})_2\text{D}_3$ and PI3K/AKT Inhibitors Cooperate to Inhibit Cell Cycle Progression and Induce Senescence

Having demonstrated synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT inhibitors, we sought to explore the mechanism. First, we performed video time-lapse microscopy analysis of DU145 cells treated with API-2 (0.25 μM) or $1\alpha,25(\text{OH})_2\text{D}_3$ (100 nM) alone as well as in combination. We did not observe any considerable apoptosis in either of the treatments (data not shown). Next we evaluated cell cycle distribution of DU145 cells treated with API-2 and/or $1\alpha,25(\text{OH})_2\text{D}_3$. Cell cycle analysis demonstrated a small but statistically significant increase in G_1 -arrested cells treated with the combination of API-2 at 0.25 μM with $1\alpha,25(\text{OH})_2\text{D}_3$ at 10 nM compared to either agent alone (Figure 7).

When we assessed the effects of the drugs on senescence in the human primary prostate cancer cell strain WFU273Ca, we found that $1\alpha,25(\text{OH})_2\text{D}_3$ was able to induce SA- β -Gal activity, which was associated with morphological features consistent with senescence (Figures 8A, 9). Interestingly, inhibition of the AKT pathway with API-2 alone induced senescence in WFU273Ca cells, with 0.5 μM API-2 causing 30% of the cells to undergo senescence, suggesting a role for activated AKT in the prevention of senescence in these cells (Figures 8B, 9). The combination of API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ cooperated to induce greater SA- β -Gal activity, which was statistically significant by ANOVA (Figures 8C, 9). Treatment with 0.1 μM API-2 induced senescence in 6.5% of WFU273Ca cells and 10 nM $1\alpha,25(\text{OH})_2\text{D}_3$ induced senescence in

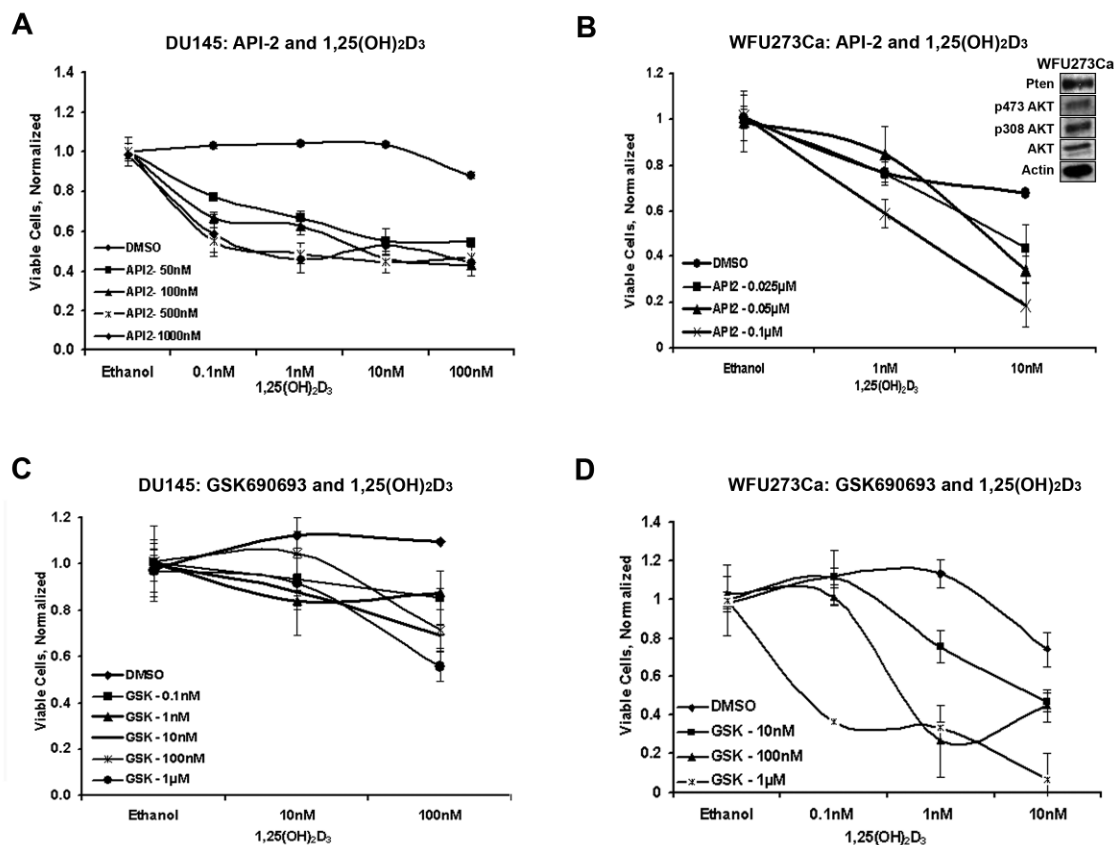


Figure 5. AKT inhibitors API-2 and GSK690693 synergize with 1 α ,25(OH)₂D₃ to inhibit growth of DU145 cells and human primary prostate cancer strain WFU273Ca. Growth inhibition of DU145 cells (A) and human primary prostate cancer cell strain WFU273Ca (B) in response to API-2 and 1 α ,25(OH)₂D₃ alone or in combination. (C) Growth inhibition of DU145 cells in response to GSK690693 and 1 α ,25(OH)₂D₃ alone or in combination. Insert demonstrates PTEN and phospho-AKT levels in WFU273Ca cell strain as determined by immunoblot. (D) Growth inhibition of human primary prostate cancer cell strain WFU273Ca in response to GSK690693 and 1 α ,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in Materials and Methods.

TABLE II.

Synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT Inhibitors***

1,25(OH)2D3, nM	AKT inhibitor, nM	CI*	DRI**		Synergism
			1α,25(OH)2D3	AKT Inhibitor	
DU145					
AKT Inhibitor: API-2					
0.1	50	0.18*10 ⁻⁶	5.5*10 ⁴	4.0*10 ⁶	Very Strong
0.1	100	0.12*10 ⁻⁶	8.7*10 ⁴	4.4*10 ⁶	Very Strong
0.1	500	7.98*10 ⁻⁶	1.3*10 ⁵	1.9*10 ⁶	Very Strong
0.1	1000	9.82*10 ⁻⁶	1.2*10 ⁵	7.4*10 ⁵	Very Strong
1	50	0.75*10 ⁻⁶	1.3*10 ⁴	1.8*10 ⁷	Very Strong
1	100	0.55*10 ⁻⁶	1.8*10 ⁴	1.6*10 ⁷	Very Strong
1	500	0.60*10 ⁻⁶	1.7*10 ⁴	2.7*10 ⁶	Very Strong
1	1000	0.54*10 ⁻⁶	1.9*10 ⁴	1.6*10 ⁶	Very Strong
10	50	0.001	872.1	8.8*10 ⁶	Very Strong
10	100	0.001	1014.8	5.7*10 ⁶	Very Strong
10	500	0.001	1950.4	3.5*10 ⁶	Very Strong
10	1000	0.001	1461.5	1.1*10 ⁶	Very Strong
100	50	0.007	135.6	1.9*10 ⁷	Very Strong
100	100	0.005	206.7	1.9*10 ⁷	Very Strong
100	500	0.006	180.3	3.1*10 ⁶	Very Strong
100	1000	0.005	193.0	1.7*10 ⁶	Very Strong
WFU275CA					
AKT inhibitor: API-2					
0.1	0.1	0.799	283.0	1.3	Moderate
1	0.05	0.720	12.2	1.6	Moderate
1	0.1	0.714	36.9	1.5	Moderate
10	0.025	0.216	9.0	9.6	Strong
10	0.05	0.169	22.5	8.0	Strong
10	0.1	0.176	52.0	6.4	Strong
DU145					
AKT inhibitor: GSK690693					
10	1	0.003	1.1*10 ⁶	391.3	Very Strong
10	10	0.070	6.5*10 ⁴	14.3	Very Strong
100	0.1	0.0001	2.3*10 ⁴	2241.4	Very Strong
100	1	0.004	3.9*10 ⁴	268.7	Very Strong
100	10	0.010	1.5*10 ⁶	96.9	Very Strong
100	100	0.061	6.6*10 ⁶	16.3	Very Strong
100	1000	0.281	6.0*10 ⁷	3.5	Strong
WFU273CA					
AKT inhibitor: GSK690693					
0.1	1000	0.773	5158.7	1.3	Moderate
1	10	0.062	37.1	28.5	Very Strong
1	100	0.120	255.9	8.7	Strong
1	1000	0.747	550.1	1.3	Moderate
10	10	0.115	10.5	51.7	Strong
10	100	0.234	14.1	6.1	Strong
10	1000	0.344	215.4	3.0	Intermediate

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism)

**Dose-reduction Index. DRI is calculated using the equation: $(DRI)_1 = (D_x)_1 / D_1$ and $(DRI)_2 = (D_x)_2 / D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. DRI values for $1\alpha,25(OH)_2D_3$ treatment in DU145 are high due to inability of the program to correctly calculate DRI based on the formula as the x effect of the $1\alpha,25(OH)_2D_3$ alone is near zero.

*** Data are from assays depicted in Figure 5, only statistically significantly different means by ANOVA are depicted.

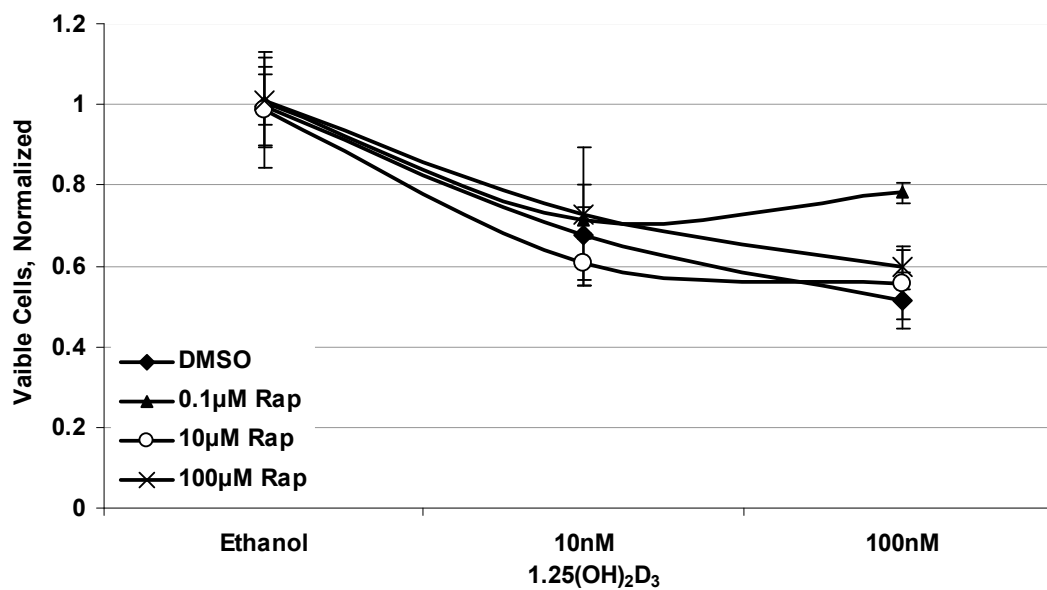


Figure 6. Inhibitor of mTOR Rapamycin and 1 α ,25(OH)₂D₃ do not synergize or cooperate to inhibit growth of DU145 cells. Growth inhibition of DU145 cells in response to Rapamycin and 1 α ,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in Materials and Methods.

7.3% of the cells; while the combination of the two led to 24.0% of the cells to undergo senescence (Figure 8C). DU145 cells also showed cooperative induction of SA- β -gal (Figure 10A).

As the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ commonly involve upregulation of p21^{Cip1} and/or p27^{Kip1} (6), we sought to test the effect of AKT inhibition and $1\alpha,25(\text{OH})_2\text{D}_3$ treatment on protein levels of p21^{Cip1} and p27^{Kip1} in DU145. The result demonstrated that 24hr of treatment with API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ cooperated to increase p21^{Cip1} protein (Figure 10B). While 0.25 μM API-2 treatment increased p21 by 3.7 fold and 100nM $1\alpha,25(\text{OH})_2\text{D}_3$ did not have a significant effect, the combination of the two led to an 8.3 fold increase in the p21^{Cip1} protein level compared to the base level. Although API-2 treatment modestly increased p27 levels, $1\alpha,25(\text{OH})_2\text{D}_3$, alone or in combination with API-2 did not demonstrate a significant effect on p27 protein levels.

Together these data suggest that synergism between API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cancer cells might be a result of cooperative induction of cell cycle arrest and senescence, possibly through induction of p21^{Cip1} levels.

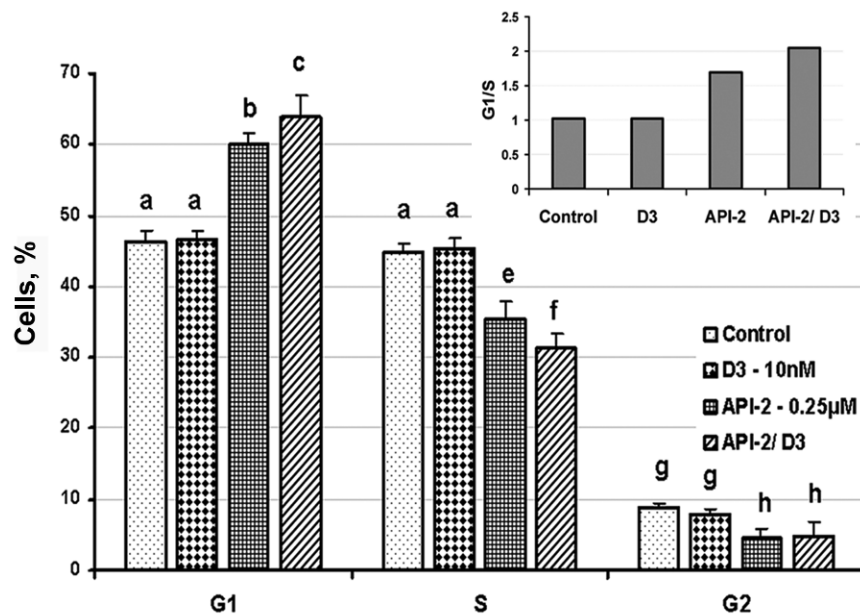


Figure 7. AKT inhibitor API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ cooperate to induce G1-arrest in DU145 cells. DU145 cells were treated with the indicated doses of API-2, $1\alpha,25(\text{OH})_2\text{D}_3$ or the combination of the two compounds for 24 hr, and evaluated for cell cycle distribution as described in the Materials and Methods section. Insert demonstrates the G1/S ratios. Values are means for the triplicates $\pm$ SD. Means without a common letter are significantly different by ANOVA ($P < 0.05$).

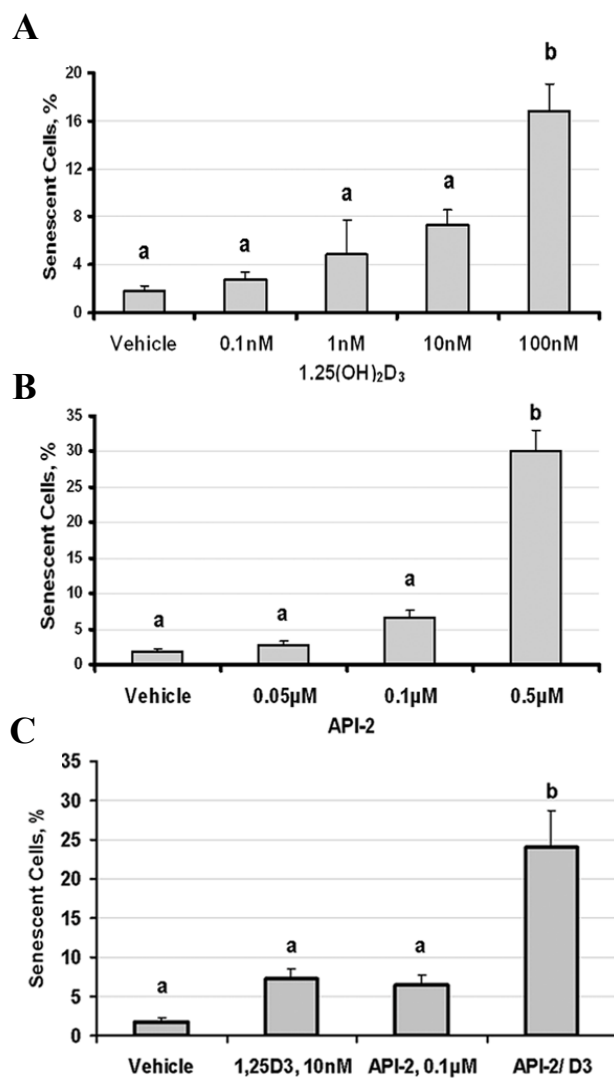


Figure 8. $1\alpha,25(\text{OH})_2\text{D}_3$ and API-2 cooperate to induce senescence in human primary prostate cancer cell strain WFU273Ca. Quantitative data from multiple images of WFU273Ca cells treated as indicated. **(A)** Induction of senescence in the WFU273Ca human primary prostate cancer cell strain treated with vehicle (ethanol) or increasing concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$. **(B)** Induction of senescence in the WFU273Ca human primary prostate cancer cell strain treated with vehicle (DMSO) or increasing concentrations of AKT inhibitor API-2. **(C)** Induction of senescence in the WFU273Ca human primary prostate cancer cell strain treated with vehicle (ethanol and DMSO), 10 nM $1\alpha,25(\text{OH})_2\text{D}_3$, 0.1 μM API-2, and the combination of 10 nM $1\alpha,25(\text{OH})_2\text{D}_3$ and 0.1 μM API-2. Analysis was performed as described in the Materials and Methods section. Means $\pm$ SE are shown. Means without a common letter are significantly different by ANOVA ($P < 0.05$).

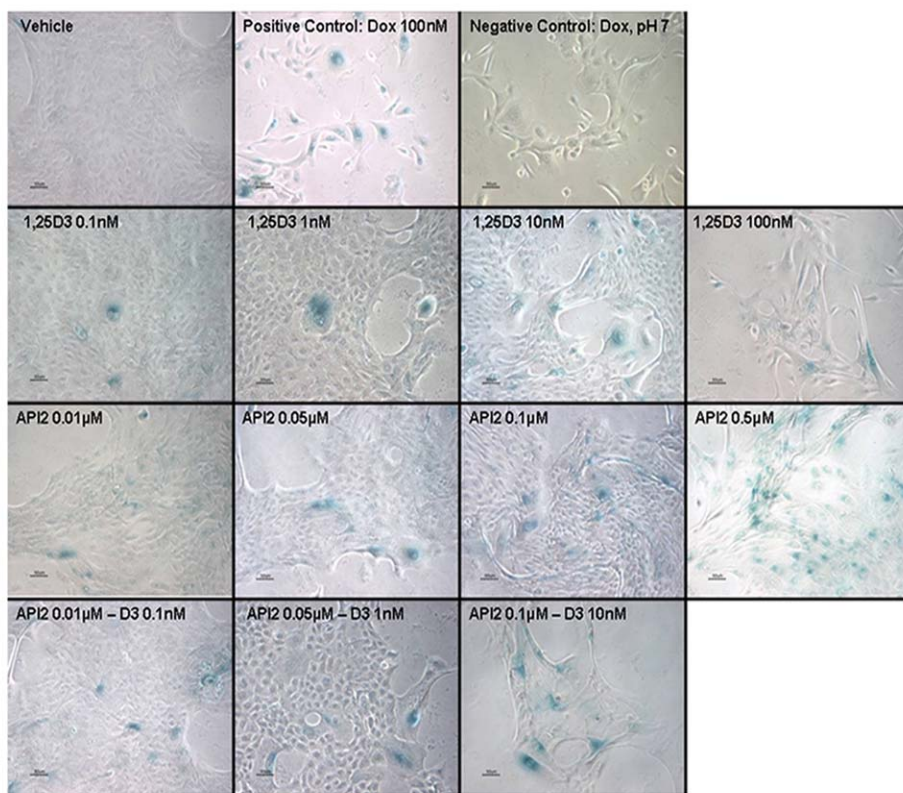
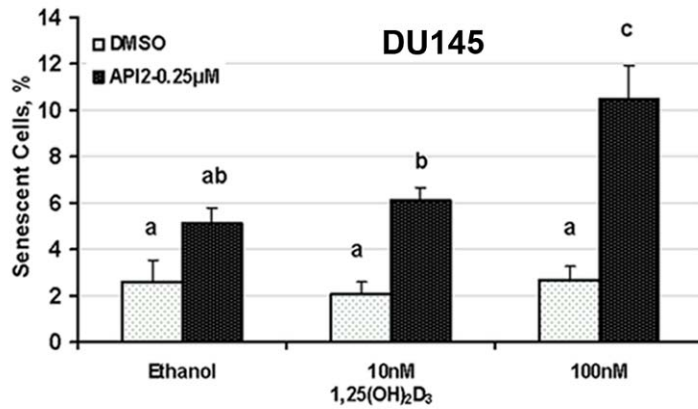


Figure 9. Representative photographs of $1\alpha,25(\text{OH})_2\text{D}_3$ and API-2 inducing SA- β -galactosidase activity in a cooperative manner in WFU273Ca cells.

A



B

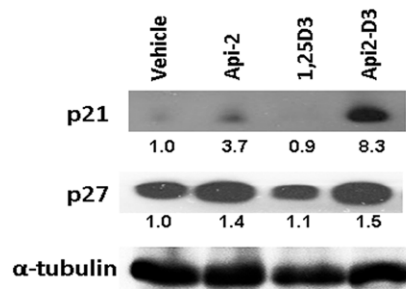


Figure 10. AKT inhibitor API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ cooperate to induce senescence and higher p21 levels in DU145 cells. (A) Induction of senescence by $1\alpha,25(\text{OH})_2\text{D}_3$ alone or in presence of $0.25\ \mu\text{M}$ API-2 in DU145 cells. Quantitative data from ten images of DU145 cells treated as indicated. Cells were grown, treated, and then SA- β -galactosidase activity was evaluated as described in the Materials and Methods section. Means $\pm$ SE are shown. Means without a common letter are significantly different by ANOVA ($P < 0.05$). **(B)** API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ cooperatively induce p21 and p27 protein levels in DU145 cells. DU145 cells were treated with vehicle, $0.25\ \mu\text{M}$ API-2 or $100\ \text{nM}$ $1\alpha,25(\text{OH})_2\text{D}_3$ or the combination of the two, and in 24 hrs protein lysates were collected and immunoblots were carried out as described in the Materials and Methods section.

2.5. DISCUSSION

In this study we showed that AKT inhibitors in combination with $1\alpha,25(\text{OH})_2\text{D}_3$ synergistically inhibit the growth of prostate cancer cells. This effect was observed with multiple inhibitors of PI3K and/or AKT in prostate cancer cell lines as well as in a primary human prostate cancer sample. Our findings might be important as AKT inhibitors (including the ones tested in this study) are being tested in clinical trials for various cancers (57,65,74), and results can quickly be translated to the clinic. However, a factor complicating the use of AKT inhibitors in clinic is that the AKT pathway presents one of the most important pathways for normal cell survival. It is not yet clear whether treatment with AKT inhibitors will have acceptable levels of toxicity at doses that are therapeutically effective.

$1\alpha,25(\text{OH})_2\text{D}_3$ used alone has been shown to have anticancer effects in a number of cancer models, but its use is associated with such toxicities as calcium mobilization at doses that are therapeutically effective (6). One strategy to overcome this problem is the creation of less calcemic $1\alpha,25(\text{OH})_2\text{D}_3$ analogs, or organizing the treatment regimen in a way that allows reduction of side-effects. Another strategy is to combine $1\alpha,25(\text{OH})_2\text{D}_3$ with other agents to develop therapeutic interventions that allow dose reduction, and thus alleviate toxicity while maintaining growth inhibitory potential. Clinical trials utilizing $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs in combination with chemotherapy in advanced prostate cancer have demonstrated the feasibility of using $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs for treatment of advanced prostate cancer (75-77). For instance, Beer et al. reported an 81% response rate for the combination of $1\alpha,25(\text{OH})_2\text{D}_3$ and docetaxel in metastatic prostate cancer, versus an expected response of 40% to 50% for docetaxel alone (75). Similarly, synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ with AKT inhibitors and the DRI values demonstrated in this study suggest that more therapeutic efficacy can be achieved by combining AKT inhibitors and $1\alpha,25(\text{OH})_2\text{D}_3$ (or its analogs), potentially reducing systemic toxicities of both compounds.

Mechanistically, combined treatment with an AKT inhibitor and $1\alpha,25(\text{OH})_2\text{D}_3$ showed no evidence of apoptosis, but moderate effects on cell cycle progression and larger effects on the induction of senescence were observed. Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ alone did not have an effect on senescence in DU145 cells, which is in agreement with the observation that these cells are not growth-inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$. However, combined with AKT inhibitor API-2, $1\alpha,25(\text{OH})_2\text{D}_3$, increased the percentage of cells undergoing senescence.

In human primary prostate cancer cell strain WFU273Ca, treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ alone was able to inhibit proliferation of the cells and also induce senescence, as demonstrated by induction of SA- β -galactosidase staining and senescence-associated cell morphology alterations. The ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to induce senescence in prostate cells has *not* been demonstrated before, and contributes to the understanding of the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cells which previously have not been clearly defined.

Senescent cells are described as cells permanently arrested in the cell cycle. These cells are refractory to proliferation stimuli, exhibit altered cell morphology and gene expression, yet remain viable and preserve metabolic activity (reviewed in (78)). There are multiple studies that have demonstrated that senescence is a mechanism that limits cellular lifespan as well as presents a barrier for cellular immortalization and progression of tumorigenesis (79,80). DNA damage or oncogene expression can induce cellular senescence, and in order to become immortal, cells have to overcome senescence by acquiring additional genetic alterations.

The process of senescence has been demonstrated to have a significant role in human cancers. For example, it was shown that human benign tumors contain senescent cells, and that these cells disappear in their malignant counterparts (81,82). In human prostate cancer, Majumder et al. (83) demonstrated that markers of cellular senescence are elevated in prostatic intraepithelial neoplasia (PIN) when compared to nondysplastic epithelial cells in the same tissue section. Chen et al. (84) showed that in specimens from early-stage human prostate cancer, markers of senescence were present in areas of prostate hyperplasia/PIN and rarely in areas of carcinoma. These findings

suggest a role for senescence as a barrier for progression of tumorigenesis in prostatic cells. The ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to induce senescence in human primary prostate cancer cell strain as demonstrated in the study, supports the use of $1\alpha,25(\text{OH})_2\text{D}_3$ for the treatment of earlier stages of human prostate cancer, with the goal of prevention of disease progression. Ultimately, combinations of $1\alpha,25(\text{OH})_2\text{D}_3$ with pharmacological AKT inhibitors might provide further benefits by stimulating senescence, reducing growth of cancer cells and blocking or slowing tumorigenesis.

It was previously demonstrated that p21 or p27 expression plays a critical role in the induction of senescence in a number of cell types (85-87) including prostate cells (83). In addition, $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to increase the steady-state levels of p27 protein in prostate cancer cells (88). Thus, we sought to explore the effect of AKT inhibition and $1\alpha,25(\text{OH})_2\text{D}_3$ treatment on p21 and p27 levels. In agreement with the lack of response to the usual antiproliferative action of $1\alpha,25(\text{OH})_2\text{D}_3$, DU145 did not induce p21 or p27 levels upon $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. However, when AKT inhibitor API-2 induced p21 protein levels, a cooperative induction of p21 by combined treatment with API-2 and $1\alpha,25(\text{OH})_2\text{D}_3$ was observed, which correlated with sensitization of the cells to the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and induction of senescence. Thus, while $1\alpha,25(\text{OH})_2\text{D}_3$ -sensitive cell strains undergo senescence upon $1,25(\text{OH})_2\text{D}_3$ treatment, the $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitive DU145 cell line required an AKT inhibitor treatment in order for $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit proliferation and induce senescence.

In summary, our results show that $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT inhibitors synergistically inhibit prostate cancer growth through induction of cell cycle arrest and senescence. These data may have implications for the clinical use of these agents in prostate cancer patients, especially in patient with high risk of disease progression.

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CHAPTER III

1 α ,25-DIHYDROXY VITAMIN D₃ SELECTIVELY INHIBITS GROWTH AND INDUCES SENESCENCE IN CELLS WITH ACUTE LOSS OF PTEN

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3.1. ABSTRACT

Loss of PTEN function and consequent activation of the AKT pathway are common events in human prostate cancer. Recent studies demonstrated that acute loss of PTEN or activation of AKT induces a senescence response in prostate cells, leading to cell-cycle arrest. Senescence-associated induction of p27 or p53 was found to be a critical step for this process, and their deletion was necessary for progression to malignancy. 1- α ,25-dihydroxy vitamin D₃ (1 α ,25(OH)₂D₃) inhibits proliferation of multiple cancer cell types including prostate cancer cells, and its antiproliferative effects involve upregulation of p27 and/or p21. Our laboratory has demonstrated that 1 α ,25(OH)₂D₃ can induce senescence in prostate cancer cells. In the present study, we sought to evaluate the effect of Pten loss on 1 α ,25(OH)₂D₃-mediated antiproliferative and pro-senescence effects. For this evaluation we used mouse prostatic epithelial cells (MPEC) with acute *in vitro* Pten loss, which was achieved by shRNA knockdown or by Cre-recombinase-mediated knockout. In both models, loss of Pten expression led to a partial induction of cellular senescence. Upon treatment with 1 α ,25(OH)₂D₃, MPEC which had lost Pten showed significantly higher levels of growth inhibition and senescence induction compared to their Pten-expressing counterparts. The results were different, however, when *Pten null* MPEC from prostate tumors were tested. *Pten null* MPEC isolated from prostates with established Pten-deletion-driven tumors represent later stages of carcinogenesis that are mediated by *Pten* loss. In contrast to the MPEC with *in vitro* acute deletion of Pten, tumor-derived *Pten null* MPEC showed a complete loss of sensitivity to 1 α ,25(OH)₂D₃-mediated growth inhibition, which was partially restored by co-treatment with a PI3K or AKT inhibitor. Together, these observations suggest that initial loss of Pten in prostate can lead to an increased sensitivity to 1 α ,25(OH)₂D₃-mediated antiproliferative effects, possibly by additive effect on senescence induction; whereas subsequent changes associated with tumor progression lead to abrogation of 1 α ,25(OH)₂D₃-sensitivity, which at least partially can be overcome by use in combination with PI3K/AKT inhibitors. Our results suggest

that $1\alpha,25(\text{OH})_2\text{D}_3$ may present an effective therapeutic option for prevention of disease progression in earlier pre-tumoral stages of prostate neoplastic disease associated with induction of senescence, while in patients with more advanced disease, its combination with PI3K/AKT inhibitors would be more advantageous.

3.2. INTRODUCTION

3.2.1. The Challenges of Treatment of Prostate Neoplastic Disease

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second most common cause of cancer death in American men (1). According to recent American Cancer Society report, PCa accounts for about 1 in every 4 newly diagnosed cancers among US men, and 1 man in 6 will be diagnosed with this disease during his lifetime (1). Since PCa rates increase with age, it can be expected that PCa might become an even greater problem as life expectancy continues to increase. As a result, PCa presents a major public health concern in the U.S. and worldwide.

A unique feature of the epidemiology of prostate cancer is the high prevalence of “incidental” (also known as “autopsy” or “subclinical”) cancer. Autopsies performed on men who died from causes other than prostate cancer demonstrate that approximately 27% of men in their 40s, 34% of men in their 50s, and 60% of men in their 80s have histological prostate cancer (2,3). Histologically, these lesions are identical to the prostate cancers that are potentially life-threatening, and are considered to represent cancers in earlier stages of the disease. Interestingly, in contrast to differences in prostate cancer mortality rates worldwide (which vary over twentyfold (4)), the prevalence of incidental prostate tumors among older men is similar regardless of their race or country of residence (5,6). These data suggest that the occurrence of subclinical prostate cancer is more ubiquitous than the clinical cancers.

For diagnosed prostate cancer, androgen-ablation is usually the first-line treatment. Initially almost all patients respond to the treatment, however, many if not all patients progress to androgen-independent prostate cancer (AIPC), in which cancer develops an ability to grow in the absence of androgens (7). AIPC is the progressive and metastatic form of PCa, and unfortunately, it is not generally amenable to current therapies.

Together, the high frequency of subclinical and clinical prostate cancer in men, consistent increases in the life-expectancy of the population, and lack of efficient treatment modalities for

advanced prostate cancer suggest that methods for the prevention of prostate cancer and for maintaining it in the subclinical state will both be essential to reducing the societal impact of this disease.

3.2.2. Vitamin D and Prostate Cancer

Vitamin D is a hormone that is synthesized through a multistep process that begins in the skin, where UV light in 270-300 nm range photocatalyzes the conversion of 7-dehydrocholesterol to cholecalciferol (vitamin D₃). Vitamin D₃ is further modified in the liver to become 25 α -hydroxy-cholecalciferol (25(OH)D₃), and then this is 1 α -hydroxylated in the kidney to become calcitriol (1 α ,25(OH)₂D₃). Actions of 25(OH)D₃ and 1 α ,25(OH)₂D₃ are limited by catabolism, primarily by 24-hydroxylase, which generates compounds that are then further metabolized to excreted products such as calcitroic acid. 1 α ,25(OH)₂D₃ is the most potent naturally occurring form of vitamin D. Although vitamin D-metabolizing enzymes are located primarily in the liver and the kidney, these enzymes are also found in many other tissues. Studies have demonstrated that tissues such as colon, breast, lung, pancreas and prostate can synthesize and degrade 1 α ,25(OH)₂D₃ (8).

In 1990, Schwartz and Hulka proposed that clinical PCa may be associated with vitamin D deficiency (9). Since this initial hypothesis suggesting a role of vitamin D in prostate cancer development, multiple laboratories have demonstrated the anticancer potential of vitamin D compounds *in vivo* and *in vitro* models, as well as in clinical trials. 1 α ,25(OH)₂D₃ has been shown to inhibit proliferation of cancer cell lines derived from breast (10), lung (11), endometrium (12), head and neck (13), hematopoietic lineages (14), and prostate (15). The ability of 1 α ,25(OH)₂D₃ to inhibit prostate cell growth has been demonstrated in primary prostatic cells, benign prostatic hyperplasia, prostate cancer, multiple prostate cancer cell lines, and in a number of xenograft models of prostate cancer (reviewed in (16)). The anticancer mechanisms for 1 α ,25(OH)₂D₃ action have been shown to include induction of cell cycle arrest, promotion of differentiation, inhibition

of proliferation, inhibition of angiogenesis, and inhibition of the invasive and migratory potential of cancer cells (16).

In most cancer cells, treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs results in accumulation of cells in the G0/G1 phase of the cell cycle (17). The regulation of cell cycle distribution by $1\alpha,25(\text{OH})_2\text{D}_3$ appears to be cell-specific, and may involve distinct pathways. $1\alpha,25(\text{OH})_2\text{D}_3$ can induce cell cycle arrest through repression of TYMS (encoding thymidylate synthetase) and TK1 (encoding thymidine kinase), activation of the INK4 family of cyclin D-dependent kinase inhibitors, downregulation of CDK2 and SKP2, or through repression of proto-oncogene MYC (18,19).

In prostate cancer cells, $1\alpha,25(\text{OH})_2\text{D}_3$ blocks cell cycle progression by acting through multiple mechanisms, however, CDK inhibitors p21 and p27 were shown to be the most common targets for $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth arrest. Thus, in LNCaP cells, $1\alpha,25(\text{OH})_2\text{D}_3$ mediates G1 arrest by increasing the expression of p21 and decreasing CDK2 activity, which was followed by a decrease in phospho-Rb levels and suppression of E2F transcriptional activity (20). The critical role of p21 in $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition was demonstrated using another prostate cancer cell line, ALVA-31, where $1\alpha,25(\text{OH})_2\text{D}_3$ increased p21 mRNA and protein levels (21). Interestingly, even though p21 is a known p53 target gene (22), p53 was not required to induce growth inhibition or G1 arrest induction by $1\alpha,25(\text{OH})_2\text{D}_3$ in LNCaP cells (23). Nevertheless, elimination of p53 function allowed the cells to recover from $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth arrest in LNCaP cells (23).

Regulation of p27 levels by $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cancer cells has been shown to occur through inhibition of p27's proteolysis (24,25). In LNCaP cells, $1\alpha,25(\text{OH})_2\text{D}_3$ downregulates the transcriptional expression of p45Skp2, which is implicated in p27 degradation, leading to reduced turnover of p27 protein (24,26). In addition, Yang and colleagues (26) showed that $1\alpha,25(\text{OH})_2\text{D}_3$ reduces nuclear levels of CDK2, which in turn reduces CDK2-mediated

phosphorylation of p27 at Thr187; since this phosphorylation is necessary for the SKP2-mediated degradation of p27, its reduction leads to increased p27 protein half-life (27,28).

In addition to cell cycle arrest mediated by $1\alpha,25(\text{OH})_2\text{D}_3$, we recently discovered a novel mechanism implicated in $1\alpha,25(\text{OH})_2\text{D}_3$ antiproliferative actions in prostate cancer cells - induction of cellular senescence (29). The mechanism of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence still remains to be determined, but it may involve p21 upregulation (29).

In the clinic, use of $1\alpha,25(\text{OH})_2\text{D}_3$ as a single therapeutic agent for the treatment of prostate cancer was evaluated in small trials, and some effectiveness was indicated by a slowing rate of PSA rise (30,31). Later clinical trials utilizing $1,25(\text{OH})_2\text{D}_3$ or its analogs in combination with chemotherapy in advanced prostate cancer have demonstrated the feasibility of using of $1,25(\text{OH})_2\text{D}_3$ for treatment of advanced prostate cancer (32-37). For instance, Beer et al. reported an 81% response rate for the combination of $1,25(\text{OH})_2\text{D}_3$ and docetaxel in metastatic prostate cancer, versus an expected response of 40% to 50% for docetaxel alone (32).

In summary, considerable *in vitro*, *in vivo* and clinical data demonstrates an anticancer potential of vitamin D compounds that could be exploited for cancer therapy and prevention.

3.2.3. PTEN and its Role in Prostate Cancer

One of the central factors in the survival of prostate cancer cells is action of the phosphoinositol-3 kinase PI3K-AKT pathway (38). Activated AKT phosphorylates a host of proteins that affect cell growth, cell cycle entry and cell survival. In prostate cancer, activation of AKT occurs most frequently due to the loss of tumor suppressor PTEN (Phosphatase and TENsin homologue deleted on chromosome 10) (39-41).

PTEN, the product of the *Pten* gene, was discovered in 1997 (42,43). PTEN is found in most tissues of the body, and its activity opposes the survival and proliferative actions of many growth factors, especially IGF (44). It is a 403 amino-acid protein with dual lipid and protein phosphatase functions that are present at the plasma membrane and in the nucleus (45), where it

keeps cellular PI-3,4,5-P3 at a low level. PTEN removes the 3'-phosphate from PI-3,4,5-P3 generated by PI3K, converting it to PI-4,5-P2 (44). Consequently, loss of PTEN is associated with activation of the PI3K/AKT/mTOR pathway (38, 46) and promotion of cellular survival and proliferation.

Structure of PTEN Protein. PTEN shares homology with the family of protein tyrosine phosphatases as well as with tensin, a cytoskeletal protein (42,43). The protein encoded by PTEN belongs to a family of dual-specificity protein phosphatases that can dephosphorylate serine, threonine and tyrosine residues (45). Protein tyrosine phosphatases can therefore antagonize and modulate the signals mediated by protein tyrosine kinases. Protein tyrosine phosphatases, including PTEN, are characterized by the presence of a signature catalytic motif, HCXXGXXRS/T (45). The integrity of the phosphatase catalytic domain and two α -helix motifs flanking the catalytic core are necessary for PTEN activity, and most of the point mutations detected in primary tumors and in cell lines are confined to exon 5 of PTEN, which encodes these domains (47).

In addition to the phosphatase domain, PTEN also contains a putative C2 regulatory domain, a PIP2 binding motif, two PEST homology regions, and a consensus PDZ binding site. The function of these domains is to control the stability and subcellular localization of PTEN, and to regulate the phosphatase activity (Figure 1) (48). As mentioned earlier, many of the cancer-related mutations of PTEN have been mapped to the conserved catalytic domain, suggesting that its tumor suppressor function is dependent on phosphatase activity (47).

Role in Disease. The PTEN tumor suppressor gene was the first phosphatase identified that is frequently mutated or deleted in various human cancers (it was also named MMAC1, for 'mutated in multiple advanced cancers') (42,43). Germline mutation in the Pten gene is shown to be associated with Cowden syndrome and the related diseases Lhermitte-Duclos disease and Bannayan-Zonana syndrome, which are characterized by the development of hyperplastic lesions (hamartomas) in multiple organs and increased risk for malignant transformation (42, 49-50).

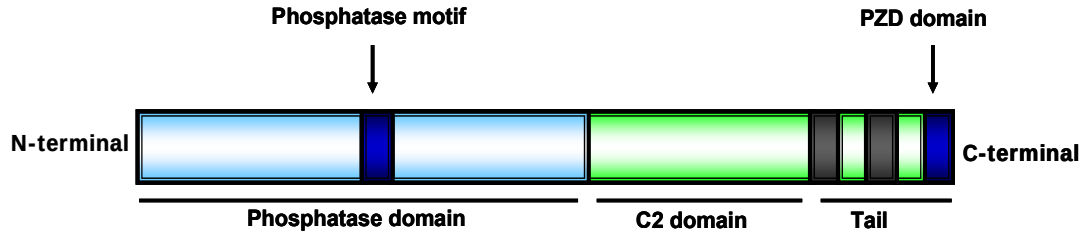


Figure 1. PTEN protein structure. PTEN has a phosphatase domain in the N-terminal region with the phosphatase motif implicated in its tumor suppressor activity. Most cancer-implicated mutations affect the phosphatase motif. The C2 domain allows for the binding of PTEN to phospholipids, possibly by effective positioning of PTEN on the membrane. The tail region contains PZD domain responsible for protein-protein interactions, and two proline-, glutamate-, serine- and threonine-rich (PEST) sequences involved in protein degradation.

PTEN is located on chromosome 10q23, a genomic region deleted in a large number of human cancers, particularly in advanced prostate cancers and glioblastomas (42,43).

Subsequently, somatic mutations or deletions of the gene were found in a large proportion of prostate, brain, breast, endometrial, skin and kidney tumors, placing PTEN among the most commonly mutated genes in human cancer (42,43,51,52).

In prostate cancer, loss of PTEN protein occurs in 20% of primary prostate tumors, and this loss is highly correlated with advanced tumor grade and stage; 60-70% of metastatic tumors exhibit a loss of PTEN protein (53,54). Moreover, loss of PTEN heterozygosity (LOH) is found in 20% to 60% of metastatic tumors (55). Thus, data suggest that advancing disease is associated with a progressive loss of PTEN or an accumulation of mutations in the PTEN gene. In addition, in prostate cancer, PTEN loss has been associated with progression to androgen independence (56), chemoresistance (57,58), radioresistance (59), bone metastasis (60) and disease recurrence after surgery (61).

Mouse Models of Pten Deletion. Recognition of the critical role of PTEN function in tumor suppression and cell biology has led to the creation of several murine models imitating the loss of PTEN expression.

It was shown that homozygous deletion of *Pten* results in early embryonic lethality, while heterozygous mice are viable, but develop hyperplastic/neoplastic changes in multiple organs as early as 4 weeks after birth, most frequently in lymphoid, endometrial, mammary, gastrointestinal and adrenal gland tissues (62,63). Most male *Pten*^{+/-} mice develop lymphoma, and at lower frequency, hyperplasia and dysplasia of several other tissues, including prostate (62-64). It has been suggested that in *Pten*^{+/-} mice, tumor development might be associated with loss of the wild-type *Pten* allele (63). *Pten*^{+/-} male mice develop prostate intraepithelial neoplasia (PIN) with nearly 100% penetrance, but these lesions apparently do not progress to macroinvasive cancers (62,65). *Pten* deletion correlates with Akt activation and growth of tumors (53), and in *Pten*^{+/-}

mice, Akt1 deficiency predictably inhibits tumor development; even Akt1 haploinsufficiency is sufficient to significantly diminish PIN formation induced by Pten deficiency (66).

To determine the consequences of prostate-specific deletion of *Pten*, mice harboring floxed alleles of *Pten* were generated and crossed with mice bearing constitutive prostate-specific expression of Cre-recombinase (ARR2PB-Cre) (67). In mice lacking both alleles of *Pten* due to expression of Cre-recombinase driven by a modified probasin promoter, PIN developed with earlier onset than in *Pten*^{+/-} mice, leading to invasive prostate cancer and ultimately to metastatic prostate cancer (53,68). Likewise, in mice with homozygous deletion of *Pten* using PSA promoter-driven Cre-recombinase, this leads to invasive prostate cancer with 100% penetrance (69). Similar pictures of progression were observed in mice bearing MMTV-Cre and *Pten* floxed alleles (70).

Monoallelic *Pten* inactivation did not lead to dramatic pathologic prostate changes in several systems (62,65), and functional synergism between Pten and other tumor suppressor genes and oncogenes was necessary for development of pathology. Heterozygous or homozygous loss of *Cdkn2b* (encoding p27^{Kip1}), *Nkx3.1* and *Ink4a/p19arf* all exacerbated the Pten prostatic phenotype. Thus, *Pten*^{+/-} mice crossed with *Nkx3.1*^{+/-} (or *Nkx3.1*^{-/-}) displayed an increase incidence of PIN lesions (71). Studies with *Pten*^{+/-};*Ink4a/Arf*^{-/-} and *Pten*^{+/-};*Cdkn1b*^{-/-} mouse models reached similar conclusions (72,73). In addition, when *Pten*^{+/-} mice were crossed with TRAMP mice, an accelerated progression of prostate cancer was observed (74).

To evaluate the role of AKT in prostate cancer development, transgenic mice with prostate-specific probasin-promoter-driven activation of AKT1 were generated through expression of a myristoylated and hence constitutively activated form of human AKT1 (Tg-AKT1) (75). In contrast to the results obtained with loss of both alleles of *Pten*, Tg-AKT1-expressing mice develop PIN phenotype without progression to invasive or metastatic cancer (75).

Taken together, the forgoing studies strongly support the role of PTEN as a tumor suppressor, with particular relevance to prostate cancer initiation and progression.

3. 2. 4. Cellular Senescence and its Induction as an Anticancer Therapy

A senescent cell is one that is permanently arrested in the cell cycle. Such cells are refractory to proliferation stimuli, exhibit altered cell morphology and gene expression, while remaining viable and preserving metabolic activity (reviewed in (76)). Many kinds of stimuli can induce a senescence response, including certain types of DNA damage, telomere shortening, strong mitogenic signals, environmental stress, sporadic inactivation of senescence suppressors and chromatin perturbations, as well as chemotherapy, radiation and hormone ablation (77-79). Senescence-associated growth arrest is best studied in human fibroblasts, and has been shown to strongly depend on activation of p53, which is usually accompanied by an increase in p21 expression (77,80,81), which in turn causes delayed induction of p16 (82,83).

Chemotherapeutic drugs generally induce both proliferation arrest and apoptosis (84). However, the stress level required for each one of these toxic responses is very different. Irreversible growth arrest can be achieved by using relatively low drug concentrations compared to those required for induction of apoptosis (85-87).

Recently, in addition to apoptosis, premature or acutely inducible senescence was found to be another drug-induced effect implicated in cancer therapy (88). It is now has been shown that cancer cells, despite multiple abnormalities, can still be forced into senescence under the action of chemotherapeutic drugs (84,89). Moreover, there is some evidence supporting the possibility that senescence programs contribute to the outcome of cancer therapy (88,90,91). For example, in the murine model of Myc-driven lymphoma, drug-treated lymphomas with apoptotic defects were still able to be forced into cytostasis, and tumors that resumed growth frequently displayed defects in either TP53 and INK4a (88). Importantly, mice bearing tumors that were susceptible to drug-induced senescence had a better prognosis following chemotherapy than those that harbored tumors with senescence defects (88). It was also shown that while BCL2 overexpression facilitates MYC-induced tumor formation by disabling apoptosis, it does not block therapy-induced senescence. Furthermore, both ARF- and p53-defficient lymphomas showed apoptotic

defects, but unlike lymphomas that lacked p53, those with no ARF were still capable of entering senescence after therapy (88). This suggests that senescence induction has potential utility in treating certain types of apoptosis-resistant cancers.

Despite the increasing evidence in mouse models that premature senescence can be induced by cancer therapy, senescence in treated human tumors needs to be measured before the general importance of senescence in cancer therapy can be confirmed. Some early evaluations have demonstrated that in human breast and lung carcinoma, senescence induced by chemotherapy is a relevant factor in determining treatment outcome (92,93). In addition, some observations in radiation therapy suggest that the induction of permanent cytostatic arrest could be the primary mode of treatment response in certain clinical cases. In particular, complete regression of prostate cancers was reported to take more than a year in some patients (94) and regression of desmoids tumors took up to two years (95) after radiation treatment. This slow course of tumor disappearance seems most consistent with radiation-induced senescence. These findings, though quite preliminary, support the possibility of senescence being implicated in response to standard chemotherapeutic regimens.

However, four factors have to be taken into account. First, it has been shown that senescent cells may produce extracellular matrix (ECM) components and secrete factors that affect growth of the neighboring cells, and also affect tissue organization (96,97). Second, it is possible that senescent cells are inducing resistance to apoptosis (98). Third, re-initiation of cell division in senescent cells is still possible under some circumstances (99). And fourth, senescent cells may persist for a long time. There are examples of senescent cells residing for years in the organism, such as the senescent melanocytes in moles or nevi (100). But this is not always the case; there are also examples of senescent cells being rapidly removed by phagocytic cells, as in the case of senescent tumor cells in liver carcinomas (101). In contrast, apoptotic cells do not persist for extended periods of time, as they are generally eliminated by phagocytes (102).

The induction of senescence as a possible mechanism for cancer treatment is a rather new hypothesis for research, and is not entirely accepted as a concept yet. However, under certain circumstances such as tumors resistant to apoptosis, senescence could possibly be a back-up mechanism (103). Identifying the genetic signatures that determine whether a tumor will benefit from induction of senescence is an important goal of pharmacogenomics. Given the increasing understanding of the networks that regulate apoptosis and senescence, it might be possible to use agents that allow for reactivation of disrupted pathways to make them more susceptible to apoptosis and senescence by chemotherapeutics.

3.2.5. Oncogene-induced Senescence

Over 10 years ago, the demonstration of a senescence-like arrest that was mediated by p53 and p16INK4a after ectopic expression of oncogenic Ras in normal primary cells introduced the concept of “oncogene-induced” senescence (104). This seminal *in vitro* observation was among the first to be validated *in vivo* using a mouse model with inducible endogenous oncogenes. Using this model, endogenous oncogenic K-Ras was shown to trigger senescence during the early stages of lung and pancreatic tumorigenesis driven by this oncogene (105). Premalignant lesions in the lung contained abundant senescent cells, whereas lung adenocarcinomas were almost completely devoid of cells positive for markers of senescence. These results were repeated with respect to Raf, a critical downstream effector of Ras-induced senescence, through use of a conditionally activated BRaf^(V600E) mouse model (106). Mice in this model developed benign lung tumors that only rarely progressed to adenocarcinoma. BRaf^(V600E) expression initially induced proliferation that was followed by growth arrest bearing certain hallmarks of senescence. Consistent with Ink4a/Arf and TP53 tumor suppressor function, BRaf^(V600E) expression combined with mutation of either Ink4a/Arf locus or TP53 led to cancer progression (106).

In addition to the above-mentioned models of oncogene-induced senescence, several groups have independently provided further experimental evidence for the occurrence of senescence resulting from oncogenic genetic manipulation. Abundant senescent cells were found in:

- (i) hyperplasias of the pituitary gland of mice with deregulated E2F activity (107);
- (ii) melanocytic lesions of UV-irradiated HGS/SF-transgenic mice (108);
- (iii) lymphocytes and in the mammary gland from N-Ras transgenic mice (109,110);
- (iv) benign prostatic lesions in mice lacking Pten (111);
- (v) PIN lesions of mice with targeted overexpression of AKT1 in the prostate (112);
- (vi) PIN lesions of mice overexpressing RHEB, which links AKT to mTOR (113);
- (vii) mouse kidney with conditional deletion of von Hippel-Lindau (Vhl) (114); and
- (viii) bladder epithelium of mice with oncogenic HRas expression from either its endogenous promoter or targeted to the bladder epithelium (115,116).

An important observation coming from these various studies is that senescence was reduced or absent from malignant tumors, even though it was observed in the corresponding pre-malignant stages of tumorigenesis. While senescent tumor cells were identified in lung carcinomas, pancreatic intraductal neoplasias, PIN lesions and melanocytic nevi, which are all pre-malignant tumors (100,105,111); senescence was absent from their corresponding malignant stages, e.g. lung carcinomas, pancreatic ductal adenocarcinomas, prostate adenocarcinomas and melanomas, respectively (105,111,117). This evidence supports a role for senescence as a barrier to tumor progression to malignancy.

Importantly, genetic manipulations canceling the senescence response were tested, and it was demonstrated that they led to progression to malignancy. Prostates from mice lacking Pten developed adenocarcinomas when combined with p53 deficiency (111). Likewise, transgenic N-Ras animals developed lymphoma after deletion of Suv39H1, a gene coding for a histone H3 lysine 9 methyl-transferase, which is believed to be involved in the *de novo* formation of

heterochromatin that takes part in senescence (109). Transgenic expression of activated AKT1 in the murine prostate induces PIN, but in combination with genetic ablation of p27 it leads to downregulation of senescence markers and progression to cancer (112).

Tumor cell senescence has not only been observed in mouse models, but also has been reported in humans. Thus, pre-malignant human colon adenomas also demonstrate senescence-associated features (118-120). Similarly, human PIN lesions express markers of senescence, which are absent in prostate cancer (111,121). Signs of senescence are also observed in neurofibromas from NF1 mutant patients, in which this genetic defect leads to constitutively high levels of Ras activity (122).

In summary, there is now compelling evidence to associate senescent cells with premalignant stages of tumor development. Up-regulation of strong cell-cycle regulators is necessary for the induction of senescence, and their loss is essential for the progression of a premalignant lesion demonstrating signs of senescence to become a malignancy. Moreover, it has now been suggested that senescence might potentially serve as a useful marker for tumor staging (123).

3.2.6. Senescence Induction as an Anticancer Mechanism in Pten-loss Model

Most human epithelial cancers progress from dysplastic *in situ* lesions to invasive and ultimately metastatic disease. PIN is a precursor of invasive prostate cancer is well-studied, however, the molecular mechanisms underlying the transition from PIN to PCa are largely unknown. Majumder and colleagues have demonstrated that transgenic expression of activated AKT1 in the murine prostate induces a PIN phenotype that does not progress to invasive prostate cancer. Expression of AKT1 transgene was associated with decreased proliferation, increased levels of p27^{Kip1} and the induction of markers of senescence (112). Loss or downregulation of p27^{Kip1} in Tg-AKT1 prostates led to increased proliferation rates, loss of senescence markers, and progression of the PIN phenotype to invasive cancer. Importantly, there have been similar findings in human prostate cancer. Markers of senescence as well as increased p27Kip1 levels

were observed in human PIN not associated with invasive cancer, unlike normal counterparts and PIN cells adjacent to invasive cancer, where the p27-mediated checkpoint loss may have already occurred (112). The authors suggested that loss of polarity in the Tg-AKT1-induced PIN led to p27-mediated checkpoint and senescence, which limited the progression of PIN to invasive cancer.

In an earlier study by Chen and colleagues (111), acute Pten inactivation also induced growth arrest and the p53-dependent cellular senescence pathway both *in vivo* and *in vitro*. While animals with Cre-mediated prostate-specific homozygous deletion of *Pten* (*Pten*^{pc/-}) presented with invasive prostate cancer after a 4-6 months latency, *Pten*^{pc/-}; *Trp53*^{-/-} animals elicited invasive prostate cancer as early as 2 weeks. *Pten*-null (*Pten*^{pc/-}) prostates of 11-week old animals contained large numbers of senescent cells (a 20-fold increase compared to WT animals), whereas *Pten*^{pc/-}; *Trp53*^{-/-} double-null animals had a marked decrease in the number of senescent cells. The same group later demonstrated that Pten-loss-induced senescence was not associated with an induction of DNA damage response (124). Furthermore, the authors detected evidence of cellular senescence in specimens from early-stage human prostate cancer, where strong SA-β-gal staining was observed in prostate hyperplasia/PIN in some glands, but never in areas flanking a tumor (111).

Taken together, the forgoing findings suggest that initial loss of PTEN or activation of AKT1 in prostate leads to PIN formation and induction of senescence, which might be associated with an increase in p27 and/or p53 levels. Induction of PIN-associated senescence might represent an earlier phase of neoplastic transformation that limits the progression to invasive prostate cancer. Additional changes allowing for elimination of the senescence response are required for the progression to malignancy.

3.2.7. Summary and Goals of the Study

As described in detail above, recent findings have identified a role for senescence as a barrier to progression of oncogene-induced neoplastic lesions to malignancy in multiple experimental systems (reviewed in (125)). In murine models of prostate cancer, loss of Pten or activation of Akt1 was associated with induction of senescence and a decrease in cellular proliferation (111,112). Moreover, human PIN was shown to be associated with increased expression of senescence markers that were absent from adjacent normal tissue as well as from the cancer area (111,112). Several proteins with well-established functions in cell-cycle regulation (e.g. 16, p21, p27, p53, Cdk2, Skp2) were shown to be implicated and often essential for oncogene-induced senescence (111,112, 126-129). Thus, it can be suggested that therapeutic agents regulating the aforementioned proteins might have utility in preventing cancer progression from *in situ* dysplasia to invasion. This is particularly relevant to prostate cancer, since as many as 30% of men with a diagnosis of PIN on biopsy are subsequently found to harbor an invasive prostatic adenocarcinoma on repeat biopsy (130).

$1\alpha,25(\text{OH})_2\text{D}_3$ has demonstrated a strong antiproliferative potential in prostate cancer cells, as well as ability to upregulate p21 and p27 levels (131), decrease Cdk2 activity (26, 131) or downregulate Skp2 levels (24, 26), all of which have been implicated in oncogene-induced senescence. Moreover, we recently demonstrated the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to stimulate senescence in human prostate cells which paralleled $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition (29).

Thus, we sought to investigate the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit proliferation and induce senescence in mouse prostate epithelial cells with lost Pten expression, which presumably represent cells in an early stage of the tumorigenesis process, as well as in Pten-null human invasive prostate cancer cells.

3.3. MATERIALS AND METHODS

Materials. $1\alpha,25(\text{OH})_2\text{D}_3$ (Sigma-Aldrich Co., St Louis, MO) was reconstituted in 100% ethanol and stored at -80°C . LY294002 (Sigma-Aldrich Co., St Louis, MO) and SH-5 (Calbiochem Co., La Jolla, CA, generously provided by Dr. George Kulik (WFU)) were reconstituted in DMSO and stored at -20°C .

MPEC with Acute Deletion of Pten. Prostate-specific Pten-knockout mice were generated by crossing $Pten^{\text{loxP/loxP}}$ mice (67) with mice of the ARR2Probasin-cre transgenic line PB-cre4, wherein the Cre recombinase is expressed under the control of a modified rat prostate-specific probasin promoter, as previously reported (132). $Pten^{\text{lox/lox}; \text{pbCre-}}$ anterior mouse prostatic epithelial cells (MPEC) were isolated from 8 week old $Pten^{\text{lox/lox}; \text{pbCre-}}$ animals (mixed C57BL/6 and BALB/c background (132,133)) by a previously described method (134). $Pten^{\text{lox/lox}; \text{pbCre-}}$ were infected with self-deleting Cre-recombinase lentivirus to generate $Pten^{-/-}$ MPEC (135). Deletion was validated by PCR and immunoblot.

shRNA Infection. WFU3 MPEC were isolated from prostates of B1/6; 129/SVEV mice as previously described (134), and were infected with lentivirus expressing shRNA targeting Pten (gaa cct gat cat tat aga tat t) or scrambled control shRNA (gggc cat ggc acg tac ggc aag). Lentivirus production and infection procedures were performed as previously described (136). MPEC were clonally selected using serial dilution (137) and Pten status was confirmed by immunoblot.

Tumor-derived Pten null MPEC. Prostate-specific Pten-knockout mice were generated by crossing $Pten^{\text{loxP/loxP}}$ mice (67) with mice of the ARR2Probasin-cre transgenic line PB-cre4, wherein the Cre recombinase is under the control of a modified rat prostate-specific probasin promoter, as previously reported (132). $Pten^{\text{lox/lox}; \text{pbCre+}}$ mice develop histologically confirmed tumors at 8 weeks of age (133). Tumor-derived *Pten null* MPEC were isolated by previously described method (134) from prostate tumors of 4 months old $Pten^{\text{lox/lox}; \text{pbCre+}}$ animals. *Pten Wt*

MPEC isolated from histologically normal prostates of *Pten*^{wt/lox; Cre-} of the same age were used as controls. Single-cell clones of *Pten null* and *Pten Wt* MPEC were established as previously described (134), and *Pten* status was validated by PCR and immunoblot.

Tissue Culture. MPEC were grown as described previously (134). Briefly, cells were plated at 10^4 cells per 35 mm dish in triplicate. In 48 hours the medium was replaced with fresh medium containing vehicle (ethanol) or increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$. Treatment was reapplied every 48 hours until cells in either of the treatment groups reached 90% confluency.

Cell Viability Assay. Cell viability was determined using the Vi-CELL XR cell viability analyzer (Beckman Coulter, Miami, FL). Trypan blue-positive cells were considered dead, and the numbers of viable cells was calculated by the analyzer according to the manufacturer's instructions. Cell viability for pooled populations of WFU3 infected with scrambled control shRNA or *Pten* shRNA, and for tumor-derived *Pten null* MPEC and *Pten Wt* MPEC, was determined using a trypan blue-exclusion assay, and the number of viable cells was counted using hemacytometer.

Clonogenic Survival Assays. Cells were plated in 60mm diameter dishes at 2000 or 4000 cells per plate in medium containing 1nM, 10nM or 100nM $1\alpha,25(\text{OH})_2\text{D}_3$ or vehicle (ethanol, 1x = 0.1% V/V), and were allowed to grow for 7 days. Platings were performed in quadruplicate. After incubation, cells were fixed with 10% methanol–10% acetic acid solution and stained with a 0.4% solution of crystal violet. Colonies with >50 cells were counted. Error bars represent the standard deviation (SD).

Senescence-associated- β -galactosidase (SA- β -gal) Activity. Cells were cultured and treated as described above. After 5-7 days of treatment, SA- β -galactosidase activity was evaluated by the method of Dimri et al. (138). For a positive control, SA- β -gal activity was evaluated in cells treated with 100 nM Doxorubicin; for a negative control, SA- β -gal activity was evaluated in cells treated with 100 nM Doxorubicin at pH 7, which inhibits SA- β -gal activity. Digital images were taken from 10 random areas at 20X magnification. Digital images were evaluated using

Photoshop CS2 9.0.2 (Adobe Systems, San Jose, CA). The number of SA- β -gal positive cells was counted in each image, and is presented as percent of total cell number $\pm$ standard error (SE).

Detection of Phosphorylated Proteins. Cells were grown to approximately 70-80% confluency, medium was replaced with fresh medium, and in 24 hours, protein lysates were prepared. Cells were washed with cold PBS, and lysis buffer was applied (1%NP-40, 50mM Tris-HCl, 150mM NaCl, 5mM EDTA with freshly added protease inhibitor cocktail, 100mM DTT, 100mM Na_3VO_4 and 500mM NaF). Cells were left on ice for 20 minutes, collected and centrifuged. Supernatants were stored at -80°C . Thirty or fifty micrograms of protein was subjected to SDS-PAGE gel electrophoresis. Proteins were transferred onto Nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocols. Membranes were incubated with appropriate dilutions of pSer473 AKT (#4051), pThr308 AKT (#2965) and AKT (#9272) antibodies (Cell Signaling Inc., Beverly, MA), and were then incubated with secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnology). Protein expression levels were detected using ECL Plus Western Blotting Detection System kit (Amersham-Pharmacia). Images were analyzed using ImageQuant TL v. 7.0 (GE Healthcare, Piscataway, NJ).

Growth Assay for Synergism Determination. Cells were plated at 10^4 cells per 35 mm dish, in triplicate. To detect synergism, cells were treated with increasing doses of LY294002, increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$ or multiple combinations of LY294002 and $1,25(\text{OH})_2\text{D}_3$. Briefly, 48 hours after plating, the cell growth medium was replaced with 1 ml of experimental medium containing twice (2x) the final concentration of LY294002 or vehicle (DMSO, 1x = 0.1% V/V). One hour later, 1 ml of medium containing twice the final concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ or vehicle (ethanol, 1x = 0.1% V/V) was added to each dish. LY294002 treatment was applied 1 hour prior to the $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. Experimental medium was applied every 48 hours. MPEC remained in the experimental medium until the vehicle control cells reached 80-

90% confluence, typically 5-7 days. Viable cells were counted with a hemacytometer after trypan blue exclusion.

Analysis of Synergism. Synergism was assessed with CalcuSyn software (Biosoft, Ferguson, MO), as previously described (139). Briefly, the dose effect for each drug alone was determined based on experimental observations using the median effect principle. The combination index (CI) for each combination was calculated according to the following equation: $CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [(D)_1(D)_2/(D_x)_1(D_x)_2]$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination, and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. $CI = 1$ represents the conservation isobologram and indicates additive effects. CI values < 1 indicates more than the expected additive effect (i.e., synergism). The Dose Reduction Index (DRI) for each drug and dose was calculated using the following equation: $(DRI)_1 = (D_x)_1/D_1$ and $(DRI)_2 = (D_x)_2/D_2$.

Statistical Analyses. Growth assays: Descriptive statistics (mean, SD, median, range) were examined for presence of outliers and distribution characteristics prior to modeling. Growth assay data were normalized to the ethanol vehicle control for $1\alpha,25(OH)_2D_3$ as a baseline, and were analyzed using two-way ANOVA. A two-sided P -value < 0.05 was considered statistically significant. SA- β -galactosidase activity: Descriptive statistics (mean, SD, median, range) were examined for presence of outliers and distribution characteristics prior to modeling. Data were analyzed using Negative Binomial count regression models where the rate of senescence was modeled with a log link, and the total number of cells (log-transformed) was used as an offset. A two-sided P -value < 0.05 was considered statistically significant. Analysis comparing fold-induction in senescence in Pten expressing MPEC versus MPEC that have lost Pten expression was performed using two-way ANOVA. All analyses were performed using SAS v9.2 (SAS Institute, Cary, NC). A two-sided p -value < 0.05 was considered statistically significant.

3. 4. RESULTS

3.4.1. Acute *in vitro* Loss of Pten Leads to Increased Sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated Growth Inhibition

To evaluate the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit growth of cells having an acute loss of Pten function, we generated mouse prostatic epithelial cells (MPEC) in which Pten expression was suppressed using an shRNA approach. MPEC WFU3 (134) were infected with lentivirus expressing scrambled control shRNA or with lentivirus expressing shRNA targeting Pten. When these two populations of MPEC were treated with increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$ we observed that MPEC infected with shRNA targeting Pten demonstrated significantly higher levels of growth inhibition compared to MPEC infected with scrambled control shRNA ($p<0.0001$) (Figure 2). MPEC infected with scrambled control shRNA were moderately growth inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$, with 100 nM of $1,25(\text{OH})_2\text{D}_3$ inhibiting about 50% of cells; whereas, in MPEC that were infected with shRNA targeting Pten, 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ inhibited growth of 96% of cells (Figure 2). To compensate for the possible heterogeneity of shRNA expression in these cells, we established single-cell clones of each of the cell types and verified Pten status of each clone, as well as phospho-AKT levels, by immunoblot (Figure 3A). In all clones tested, Pten shRNA expression led to undetectable levels of Pten protein, accompanied by an increase in phospho-Ser473 and phospho-Thr308 Akt levels (Figure 3A).

We then selected two clones of MPEC infected with lentivirus expressing scrambled control shRNA (marked as “Control clones”) having the lowest phospho-Akt levels, and two clones of MPEC infected with lentivirus expressing shRNA targeting Pten (marked as “Pten shRNA clones”) having the highest phospho-Akt levels, for further experiments. These clones were treated with increasing concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$, and cell viability was assessed by trypan-blue exclusion assay. We observed that the Pten shRNA clones demonstrated significantly higher levels of growth inhibition than the Control clones (overall $p<0.0001$, two-way ANOVA)

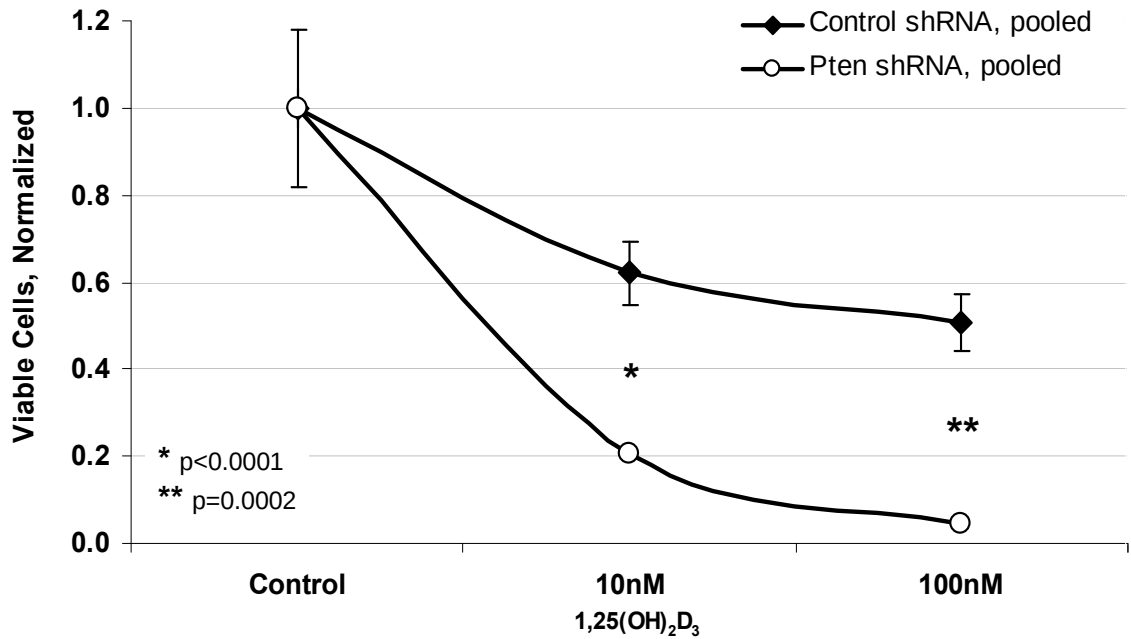
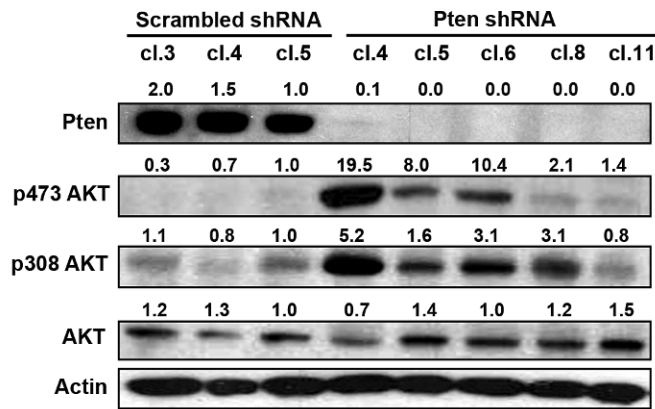


Figure 2. *In vitro* shRNA-mediated knockdown of Pten renders WFU3 MPEC more sensitive to 1 α ,25(OH)₂D₃-mediated growth inhibition. Growth inhibition of pooled populations of WFU3 MPEC infected with Pten shRNA or scrambled control shRNA in response to 1 α ,25(OH)₂D₃. Cells were grown and treated as described in the Materials and Methods section. Values are means for the triplicates $\pm$ SD. Two-way ANOVA: overall $p<0.0001$, 10 nM $p<0.0001$, 100 nM $p=0.0002$.

A



B

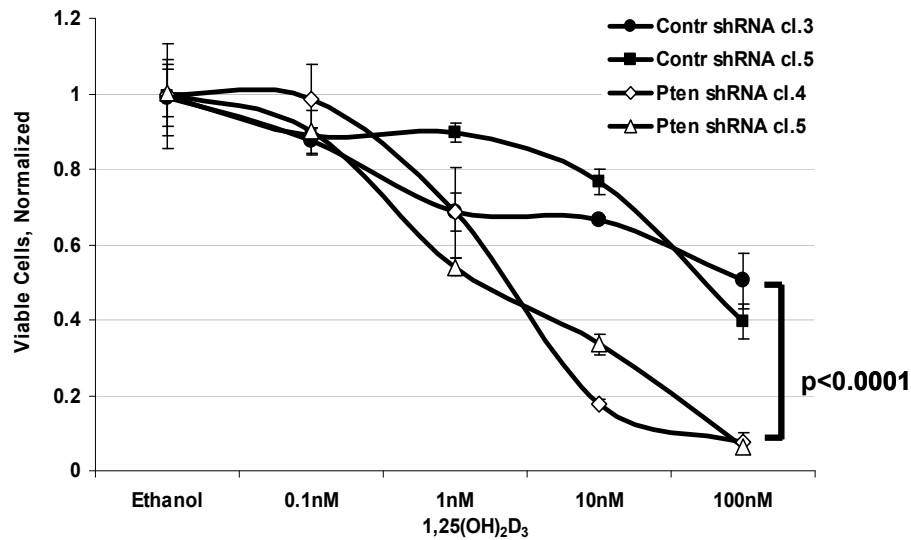


Figure 3. *In vitro* shRNA-mediated knockdown of Pten renders single-cell clones of WFU3 MPEC more sensitive to 1 α ,25(OH)₂D₃-mediated growth inhibition. **A.** Pten and phospho-Akt levels of single-cell clones of WFU3 MPEC infected with lentivirus expressing scrambled shRNA (control clones) or shRNA targeting Pten (Pten shRNA clones) as determined by immunoblot. **B.** Growth inhibition of single-cell clones of WFU3 MPEC infected with Pten shRNA or scrambled control shRNA in response to 1 α ,25(OH)₂D₃. MPEC were established and grown as described in Materials and Methods. Values are means for the triplicates $\pm$ SD. Two-way ANOVA: overall $p < 0.0001$, 0.1nM $p = 3084$, 1nM $p = 0.0004$, 10nM $p < 0.0001$, and 100nM $p < 0.0001$.

which is consistent with the $1\alpha,25(\text{OH})_2\text{D}_3$ effect in the pooled populations (Figure 2B). Thus, 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ inhibited growth of 50% to 60% of Control MPEC, and 93% to 94% of the Pten shRNA MPEC. Assessment of clonogenic growth also demonstrated higher sensitivity of clones with lost Pten expression to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition compared to their Pten-expressing counterparts (Figure 4).

Since gene suppression using an shRNA approach has the potential disadvantages of incomplete suppression of target gene expression and possible off-target effects, we sought to establish a cell line with acute *in vitro* deletion of Pten. We therefore isolated *Pten*^{lox/lox} MPEC from prostates of *Pten*^{lox/lox} but Cre-recombinase-negative animals, and infected them with lentivirus expressing self-deleting Cre-recombinase (135). PCR analysis (data not shown) and immunoblot analysis (Figure 5A) confirmed efficient knockout of Pten protein in these MPEC. Deletion of Pten led to a 5.6 fold increase in phosphor-Ser473 Akt levels, confirming activation of the Akt pathway (Figure 5A). The *Pten*^{lox/lox} and *Pten*^{-/-} (*Pten*^{lox/lox} infected with Cre-recombinase lentivirus) MPEC were treated with increasing concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$. Similar to the results obtained using shRNA-mediated Pten deletion, MPEC with Cre-mediated Pten deletion were growth-inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$ to a higher degree than the *Pten*^{lox/lox} MPEC (overall p=0.0002, two-way ANOVA) (Figure 5B). Interestingly, we observed that treatment with 1 nM and 10 nM $1\alpha,25(\text{OH})_2\text{D}_3$ led to growth stimulation of *Pten*^{lox/lox} MPEC, while these concentrations inhibited growth of *Pten*^{-/-} MPEC. *Pten*^{lox/lox} MPEC were growth inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$ only at the higher dose of $1\alpha,25(\text{OH})_2\text{D}_3$. Assessment of clonogenic growth of *Pten*^{lox/lox} and *Pten*^{-/-} MPEC demonstrated similar results, with *Pten*^{-/-} MPEC demonstrating significantly higher clonogenic growth inhibition than *Pten*^{lox/lox} MPEC, especially at lower doses of $1\alpha,25(\text{OH})_2\text{D}_3$ (Figure 6). Taken together, these findings and the findings using shRNA mediated Pten deletion demonstrate that acute *in vitro* loss of Pten expression is associated with increased sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition in mouse prostate cells.

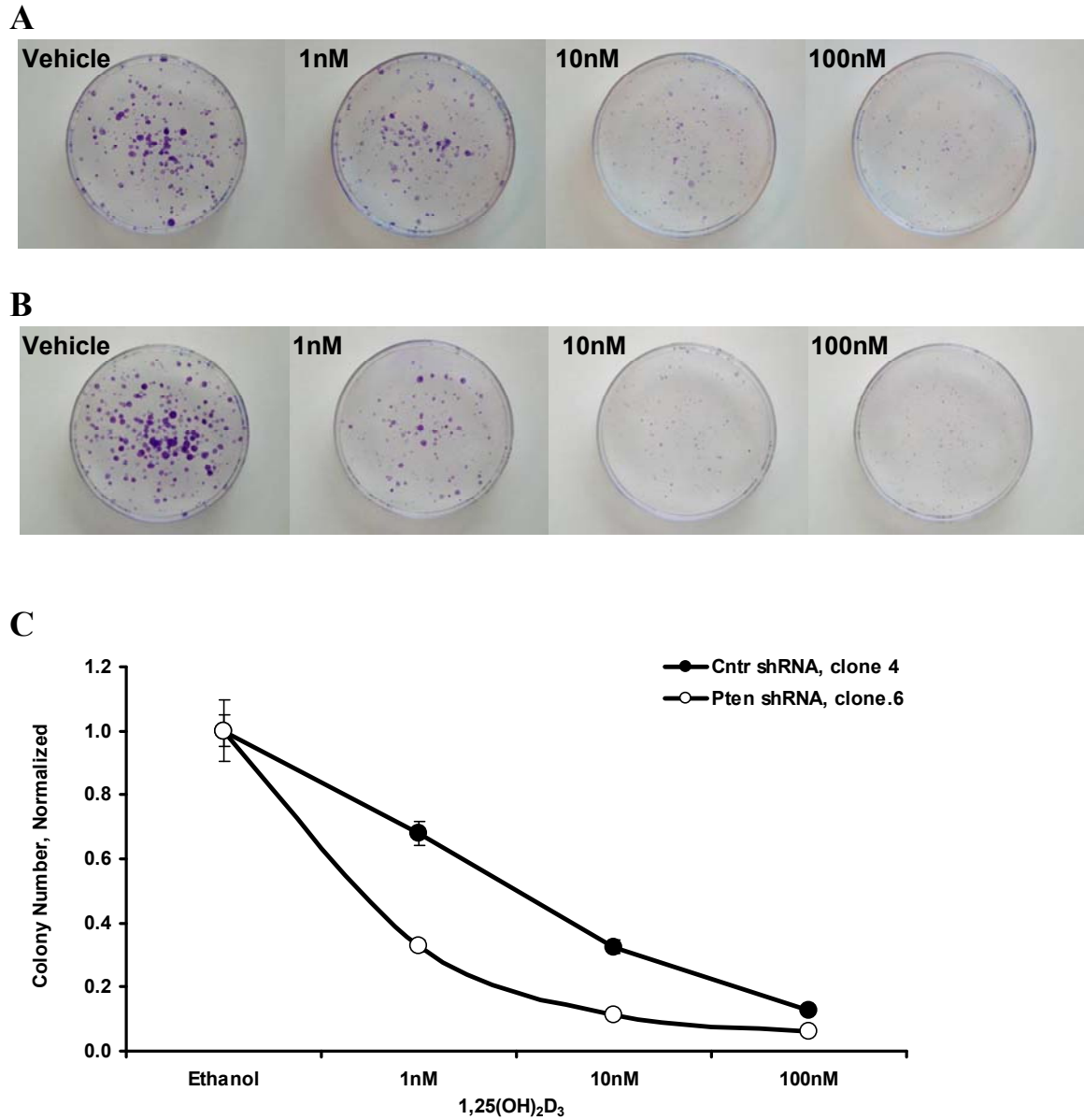
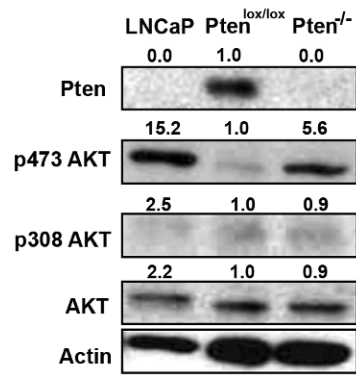


Figure 4. Clonogenic growth of single-cell clones of WFU3 MPEC is inhibited by 1 α ,25(OH)₂D₃ to a higher degree in cells with a knockdown of Pten expression.

Representative photographs of clonogenic survival of WFU3 MPEC infected with scrambled shRNA (Control shRNA, clone 4) (**A**) and WFU3 MPEC infected with shRNA targeting Pten (Pten shRNA, clone 6) (**B**) treated with increasing concentrations of 1 α ,25(OH)₂D₃. MPEC were established, grown and treated as described in the Materials and Methods section. (**C**) Quantification of clonogenic survival of Control shRNA, clone 4 and Pten shRNA, clone 6 MPEC. Values are average for quadruplicates $\pm$ SD are demonstrated. Two-way ANOVA: overall $p < 0.0001$; 1 nM $p < 0.0001$; 10 nM $p < 0.0001$; 100 nM $p = 0.002$.

A



B

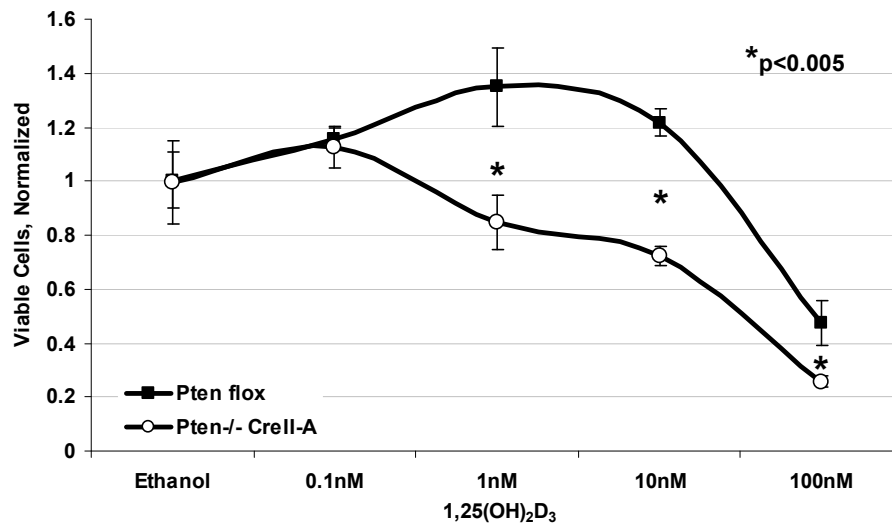


Figure 5. MPEC with *in vitro* acute deletion of *Pten* are more sensitive to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition. **A.** *Pten* and phospho-Akt levels of *Pten*^{lox/lox} and *Pten*^{-/-} MPEC as determined by immunoblot. *Pten*^{lox/lox} MPEC were isolated from prostates of *Pten*^{lox/lox} Cre-recombinase negative animals and infected with lentivirus expressing self-deleting Cre-recombinase (*Pten*^{-/-}). Protein lysate from LNCaP cells was used as control. **B.** Growth inhibition of *Pten*^{lox/lox} and *Pten*^{-/-} MPEC in response to $1\alpha,25(\text{OH})_2\text{D}_3$. Values are means for the triplicates $\pm$ SD. Two-way ANOVA: overall $p=0.0002$; 0.1nM $p=0.7252$; 1nM $p<0.0001$; 10nM $p<0.0001$, and 100nM $p=0.0040$.

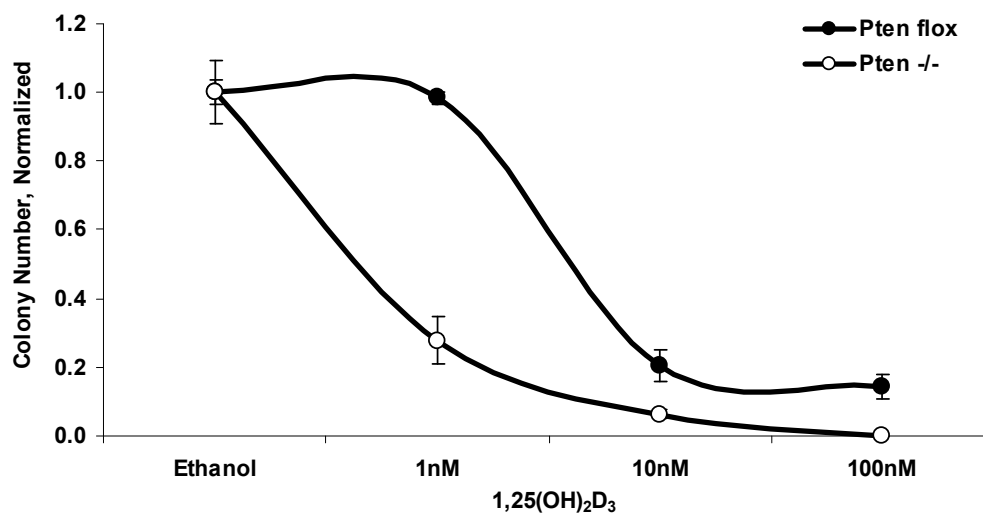


Figure 6. Clonogenic growth MPEC is inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$ to a higher degree in cells with acute deletion of *Pten*. Clonogenic growth of *Pten*^{lox/lox} and *Pten*^{-/-} MPEC treated with increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$. *Pten*^{lox/lox} MPEC isolated from prostates of *Pten*^{lox/lox} Cre-recombinase negative animals and infected with lentivirus expressing self-deleting Cre-recombinase to generate *Pten*^{-/-} MPEC. Values are means for the quadruplicates $\pm$ SD. Two-way ANOVA: overall $p < 0.0001$; 1 nM $p < 0.0001$; 10 nM $p < 0.0001$; 100 nM $p < 0.0001$.

3.4.2. Acute *in vitro* Loss of *Pten* Leads to Increased Sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated Growth Senescence

It has been shown that in mouse prostate cells, homozygous loss of *Pten* leads induction of senescence (111,112). In addition, we previously demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ can stimulate senescence in human prostate cancer cells (29). Thus, we wanted to determine whether acute loss of *Pten* expression in MPEC would affect the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to induce senescence. Therefore, MPEC with acute deletion of *Pten* mediated either by the shRNA approach or expression of Cre-recombinase, as well as their respective *Pten*-expressing control MPEC, were treated with increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$, followed by evaluation of SA- β -galactosidase activity. In agreement with previous findings (111,112), we found that *Pten* loss by either method increased the number of cells staining positive for SA- β -galactosidase (Figures 7, 9). In two of the control shRNA MPEC clones, 2.6% (clone 3) and 2.7% (clone 5) of MPEC demonstrated senescence-associated morphology and SA- β -gal-positivity; whereas in *Pten* shRNA clones, 9.6% (clone 4) and 8.0% (clone 5) of MPEC were identified as senescent (Figures 7, 8). Similarly, the *Pten*^{lox/lox} MPEC culture contained 1.7% SA- β -gal-positive cells, while 10.8% of *Pten*^{-/-} MPEC were positive for this senescence marker (Figure 9, 10).

When the shRNA approach was used to delete *Pten*, $1\alpha,25(\text{OH})_2\text{D}_3$ was able to induce senescence both in *Pten*-expressing control shRNA-treated cells and cells with shRNA-lost *Pten* expression, albeit to a different extent. Apparently reflecting the initially higher level of senescence in MPEC with lost *Pten*, a higher percentage of the cells that lost *Pten* were SA- β -gal-positive upon treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ compared to their *Pten*-expressing counterparts. In Control shRNA MPEC clones that were treated with 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$, 18.7% (clone 3) and 12.5% (clone 4) showed senescence-associated morphological changes and were positive for SA- β -gal. This same treatment induced 41.2% and 28.2% of *Pten* shRNA clone 4 and clone 5 MPEC, respectively, to undergo senescence (Figure 7).

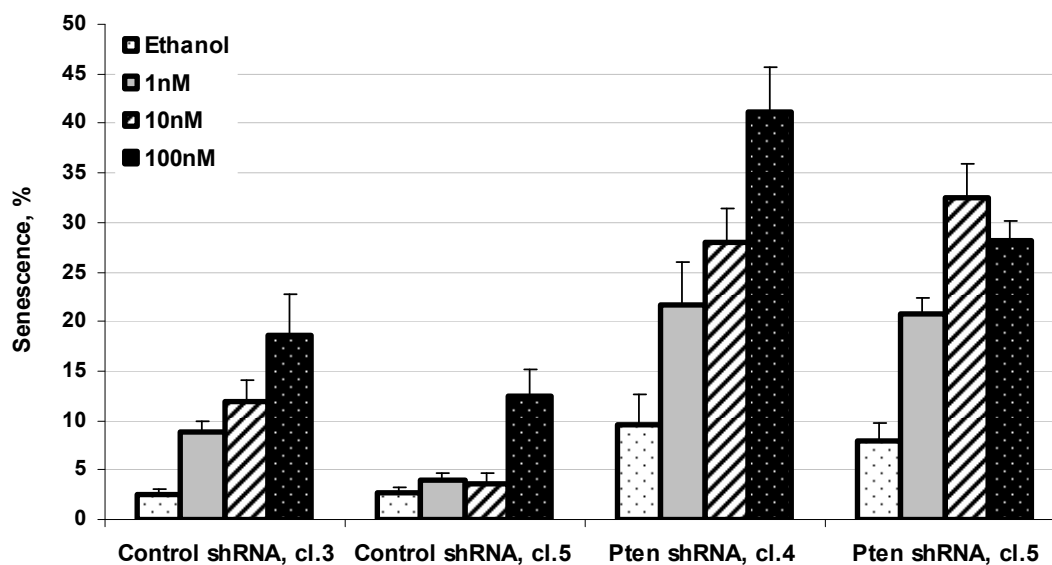


Figure 7. $1\alpha,25(\text{OH})_2\text{D}_3$ induced senescence in higher percentages of the MPEC with shRNA knockdown of Pten expression. SA- β -galactosidase activity in MPEC infected with Pten shRNA or Control (scrambled) shRNA treated with $1\alpha,25(\text{OH})_2\text{D}_3$. Cells were grown and treated, and SA- β -galactosidase activity was evaluated, as described in the Materials and Methods section. Means $\pm$ SE are shown. Statistical analysis is presented in the Table I.

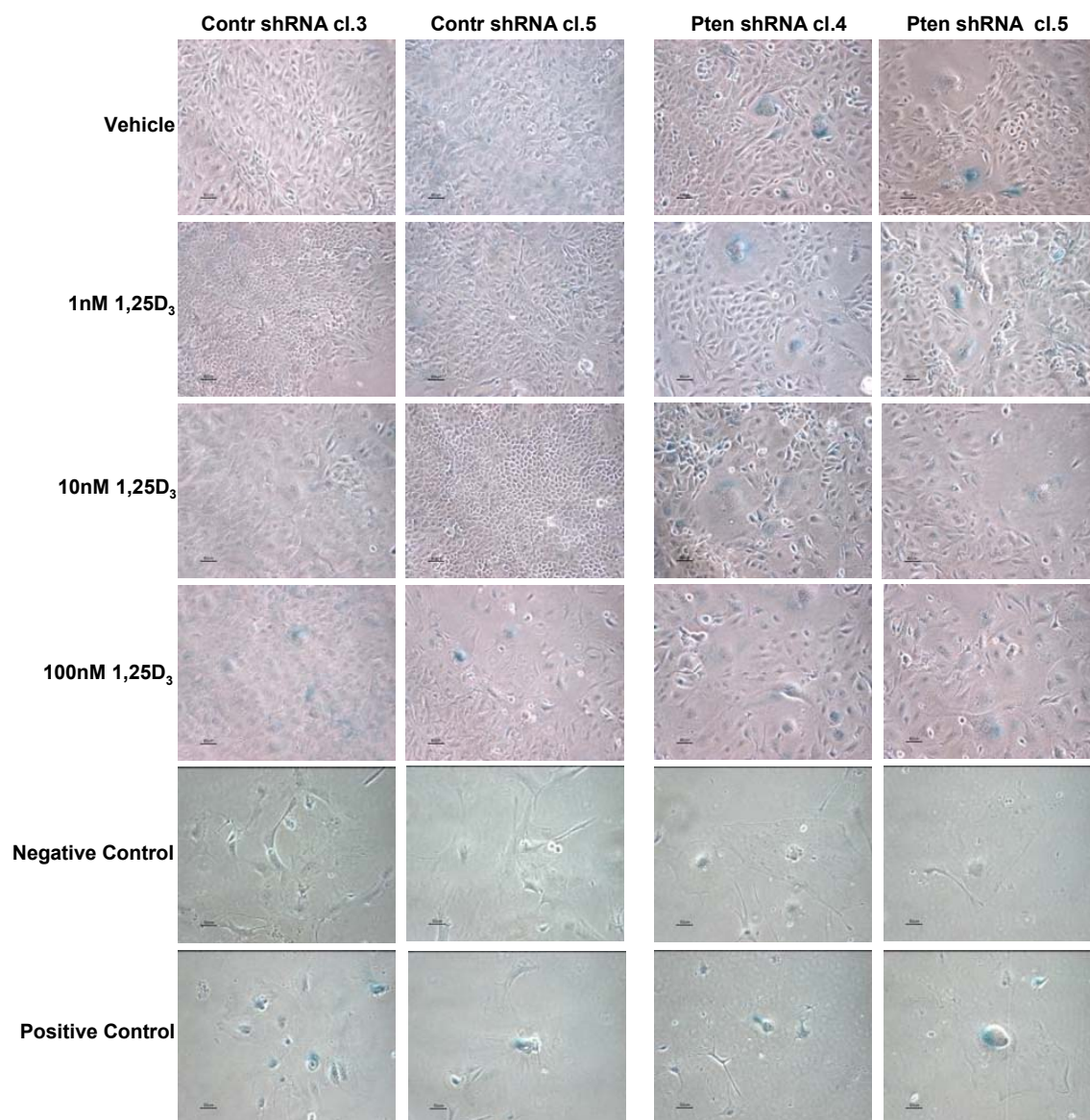


Figure 8. Representative photographs of SA-β-gal activity induced by 1α,25(OH)₂D₃ treatment in MPEC infected with Pten shRNA or Control (scrambled) shRNA. Cells were cultured and treated as described in the Materials and Methods section. SA-β-galactosidase activity was evaluated by the method of Dimri et al. (138). For positive controls, SA-β-gal activity was evaluated in cells treated with 100 nM Doxorubicin; for negative controls, SA-β-gal activity was evaluated in cells treated with 100 nM Doxorubicin at pH 7, which inhibits SA-β-gal activity.

In order to assess the impact of initial senescence levels, we compared the degree of senescence induction by $1\alpha,25(\text{OH})_2\text{D}_3$ after adjustment initial senescence levels in both the *Pten*-expressing and non-expressing shRNA-treated MPEC. The fold-induction of senescence caused by $1\alpha,25(\text{OH})_2\text{D}_3$ with and without *Pten* expression was dependent on the dose (Table I). However, the higher levels of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence in MPEC with lost *Pten* seems to be a reflection of the initial higher senescence levels in these cells. We did not observed any consistent differences between fold-induction of senescence by $1\alpha,25(\text{OH})_2\text{D}_3$ in Control shRNA-expressing cells versus *Pten* shRNA-expressing cells after the results were adjusted for the initial senescence levels in the untreated cells (Table I).

When *Pten* is deleted by the Cre-recombinase approach, the results are more compelling. In *Pten*^{lox/lox} MPEC, only the highest dose of $1\alpha,25(\text{OH})_2\text{D}_3$ tested, 100 nM, was able to stimulate senescence, leading to 8.5% of cells exhibiting senescence-associated phenotype and testing SA- β -positive, which is comparable to the levels of senescence in untreated *Pten*^{-/-} MPEC. In contrast to *Pten*^{lox/lox} MPEC, *Pten*^{-/-} cells showed a dose-dependent (but saturable) increase in the SA- β -gal activity upon $1\alpha,25(\text{OH})_2\text{D}_3$ treatment, with 10 nM $1\alpha,25(\text{OH})_2\text{D}_3$ inducing senescence in 34.0% of the cells (Figure 9). When this data was adjusted for initial senescence levels (Table 1), there was still a greater effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on *Pten*^{-/-} cells, except at the highest dose, where the fold-induction of senescence on *Pten*^{lox/lox} was greater.

3.4.3. Tumor-derived *Pten* null MPEC are not Growth-inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$

Next, we sought to test whether malignant cells, which presumably have already overcome the initial senescence response induced by *Pten* loss, would demonstrate lower sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition. As described in Chapter II, it has been shown that *Pten* loss induces senescence in mouse prostate cells by inducing p27 or p53; and that when p27 or p53 is then eliminated, the cells are able to overcome growth arrest, increase proliferative potential and form a malignant tumor (111,112). Cell lines used in our studies described above

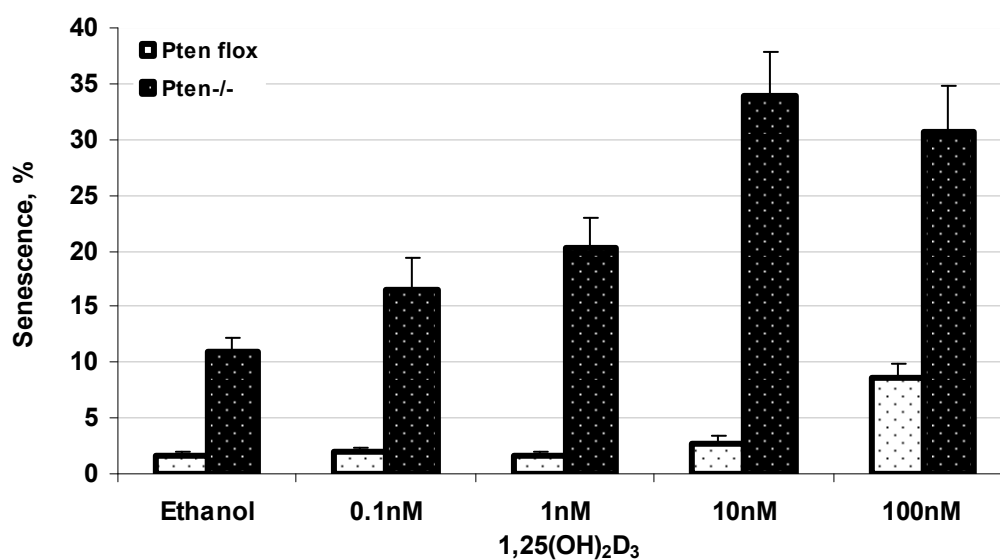


Figure 9. $1\alpha,25(\text{OH})_2\text{D}_3$ induced senescence in higher percentages of the MPEC with acute Cre-recombinase-mediated deletion of *Pten*. SA- β -galactosidase activity in *Pten*^{lox/lox} and *Pten*^{-/-} MPEC, where acute *Pten* loss was induced *in vitro* by Cre-recombinase, upon treatment with increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$. Cells were grown and treated, and (SA)- β -galactosidase activity was evaluated, as described in the Materials and Methods section. Means $\pm$ SE are shown. Statistical analysis presented in the Table I.

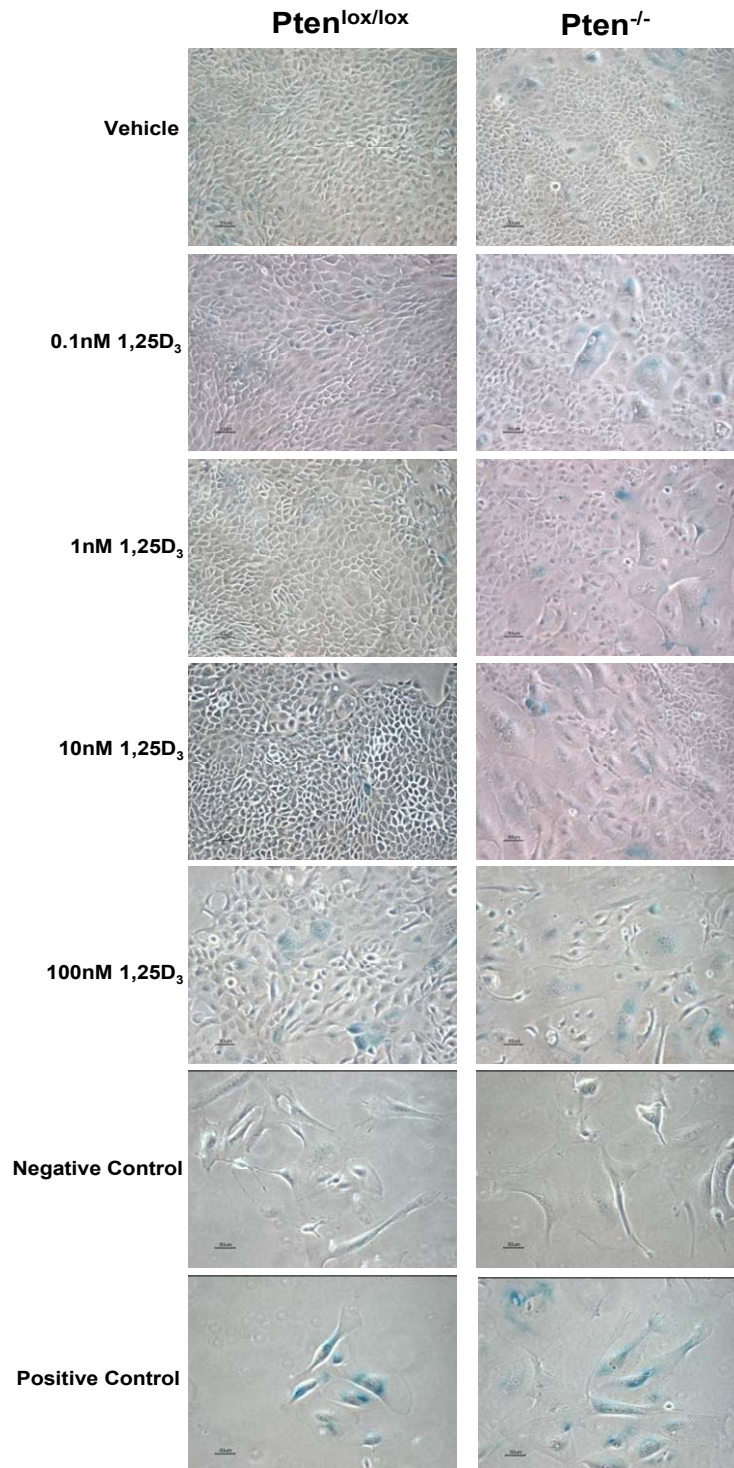


Figure 10. Representative photographs of SA-β-gal activity induced by 1 α ,25(OH)₂D₃ treatment in *Pten*^{lox/lox} and *Pten*^{-/-} MPEC. Cells were cultured and treated as described in Materials and Methods. SA-β-galactosidase activity was evaluated by the method of Dimri et al. (138). For positive controls, SA-β-gal activity was evaluated in cells treated with 100 nM Doxorubicin; for negative controls, SA-β-gal activity was evaluated in cells treated with 100 nM Doxorubicin at pH 7, which inhibits SA-β-gal activity.

TABLE I

Statistical analysis for the experiments presented in Figures 7 and 9*

	Pten flox	Pten^{-/-}	p-value
	fold induction / (%)	fold induction / (%)	fold induction / (%)
Vehicle	1.0 (1.7%)	1.0 (10.8%)	N/A / p<0.0001
0.1 nM	1.2 (2.0%)	1.5 (16.6%)	0.6467 / p<0.0001
1 nM	0.99 (1.6%)	1.87 (20.4%)	0.1845 / p<0.0001
10 nM	1.58 (2.6%)	3.1 (34.0%)	0.0167 / p<0.0001
100 nM	5.14 (8.5%)	2.8 (30.7%)	0.0002 / p<0.0001

	Contr shRNA clones	Pten shRNA clones	p-value
	fold induction / (%)	fold induction / (%)	fold induction / (%)
Vehicle	1.0 (2.6%)	1.0 (8.8%)	N/A / p<0.0001
1 nM	2.5 (6.5%)	2.4 (21.7%)	0.1355 / p<0.0001
10 nM	3.0 (7.8%)	3.5 (30.2%)	0.8753 / p<0.0001
100 nM	6.0 (15.6%)	3.9 (34.7%)	0.0156 / p<0.0001

* Data presented as percentage of SA- β -gal-positive cells before and after adjustment for the initial levels of SA- β -gal activity in Pten-expressing and non-expressing MPEC. Evaluations were done as described in the Materials and Methods section.

were isolated from histologically normal prostates and were forced to lose *Pten* *in vitro*, and thus are more representative of the pre-tumoral stage; to test the sensitivity of malignant cells to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition, we needed tumor-derived cells with non-expression of *Pten*.

For establishing tumor-derived *Pten*^{-/-} MPEC, we used prostate-specific *Pten* knockout mice that were generated by crossing *Pten*^{lox/lox} mice (67) and the ARR2Probasin-cre transgenic line PB-cre4, wherein the Cre recombinase is controlled by a modified rat prostate-specific probasin promoter (140) as previously described (132). The 8 weeks old male *Pten*^{lox/lox; pb-Cre+} (*Pten null*) mice develop either histologically-confirmed prostate tumors *in situ* or invasive carcinomas (133). Anterior prostates containing tumors were isolated from 4 months old animals, and *Pten null* MPEC were established using the method developed in our laboratory (134). These *Pten null* MPEC present later stages of tumorigenesis, and theoretically had overcome initial *Pten*-loss-mediated induction of senescence by suppressing proteins involved in the induction or maintenance of the senescence phenotype.

MPEC isolated from *Pten*^{wt/lox}; Cre-negative animals of the same genetic background and age were used for isolation of *Pten Wt* MPEC to serve as controls. *Pten Wt* and *Pten null* MPEC were clonally selected using serial dilution method and *Pten* status was confirmed by PCR as well as by Western Blot (data not shown).

Pten Wt and *Pten null* clones were treated with vehicle (ethanol) or increasing concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$, and the number of viable cells was determined by trypan blue exclusion assay. While *Pten Wt* clones treated with $1\alpha,25(\text{OH})_2\text{D}_3$ showed a dose-dependent inhibition of growth, the same concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ did not inhibit growth of *Pten null* clones, and in some clones marginally stimulated growth (Figure 11). This observation suggests that additional events in the tumorigenesis process subsequent to loss of *Pten* led to abrogation of the response by tumor-derived *Pten null* MPEC to anti-proliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$.

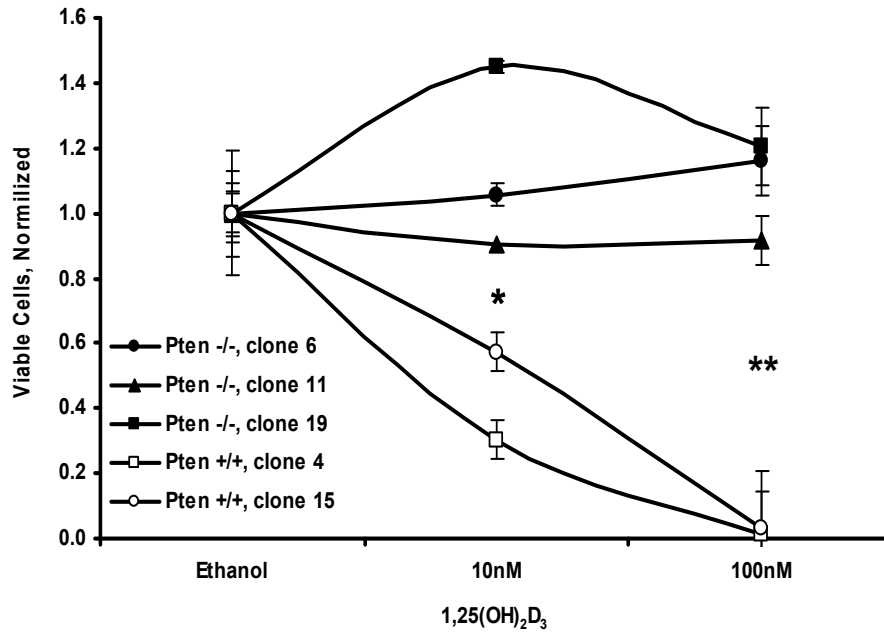


Figure 11. Tumor-derived *Pten null* MPEC clones are not growth inhibited by 1 α ,25(OH)₂D₃. MPEC were established and grown as described in the Materials and Methods section. Interaction between *Pten* status and 1 α ,25(OH)₂D₃ dose was analyzed using an ANOVA (p=0.003). * p=0.004; ** p<0.001.

3.4.4. Inhibition of PI3K/AKT Partially Restores Sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated

Growth Inhibition of Tumor-derived *Pten null* MPEC Leading to Synergistic Growth

Inhibition

Although *Pten null* MPEC isolated from tumors were not growth-inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$, combination of $1\alpha,25(\text{OH})_2\text{D}_3$ with the PI3K or AKT pathway inhibition was able to partially restore sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition in *Pten null* MPEC (Figure 12). A classical PI3K inhibitor – LY294002 (which acts on the ATP-binding site of the enzyme (141)) and an AKT inhibitor – SH-5 (a phosphatidylinositol analog that inhibits the activation of AKT (142)) were used in these experiments. While treatment with 100nM $1\alpha,25(\text{OH})_2\text{D}_3$ alone did not affect growth of *Pten null* MPEC and LY294002 alone reduced the number of viable cells by 46%, the combination of 10 μ M LY294002 with 100nM $1\alpha,25(\text{OH})_2\text{D}_3$ caused a reduction in the number of viable cells by 70%. Similarly, 100nM $1\alpha,25(\text{OH})_2\text{D}_3$ or 10 μ M SH-5 itself did not affect growth of the *Pten null* MPEC, but 100nM $1\alpha,25(\text{OH})_2\text{D}_3$ in the presence of SH-5 reduced the number of viable cells by 30%. This data reveals that inhibiting PI3K or AKT in tumor-derived *Pten null* tumor cells leads to sensitization to the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated antiproliferative response (Figure 12).

We have demonstrated (for details see Chapter II and (29)) that pharmacological inhibition of the PI3K or AKT pathways synergizes with $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit growth of prostate cancer cell lines and human primary prostate cancer cell strains, which is at least partially attributed to synergistic induction of senescence. Thus, we tested whether inhibition of the PI3K pathway synergizes with $1\alpha,25(\text{OH})_2\text{D}_3$ treatment to inhibit the growth of $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitive tumor-derived *Pten null* MPEC. Treatment of *Pten null* MPEC with increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$ or LY294002 or with multiple combinations of the two compounds led to statistically significant synergism between the two compounds at higher dose of LY294002 (10 μ M) (Figure 13, Table II).

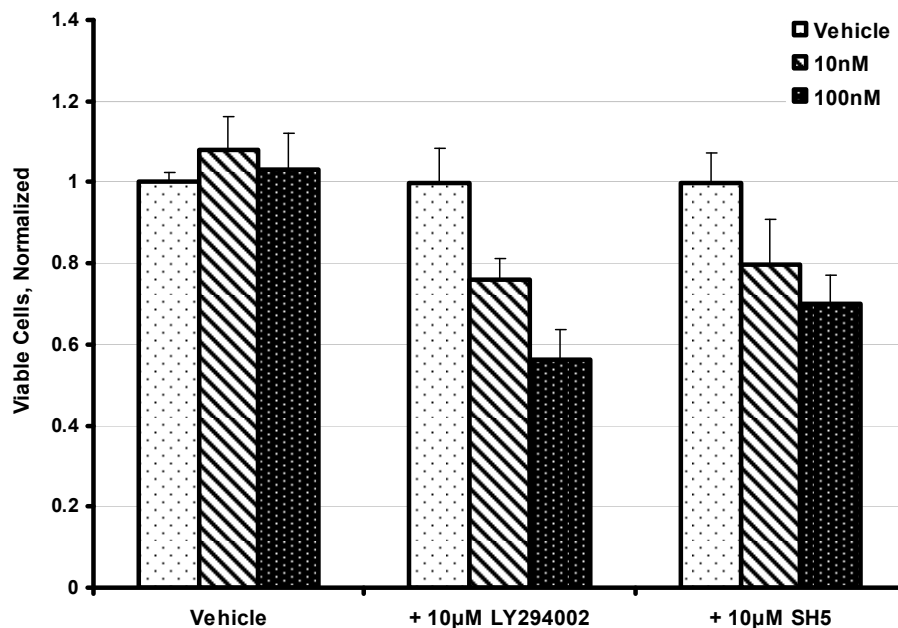


Figure 12. Inhibition of PI3K or AKT partially restores sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition of tumor-derived *Pten null* MPEC. Tumor-derived *Pten null* MPEC (clone 11) were plated, and in 24 hrs growth medium was replaced with experimental medium containing either DMSO, or 10 µM LY294002 or 10 µM SH-5. One hour after treatment with PI3K/AKT inhibitor, ethanol or 10 nM or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ was added to the medium. Experimental medium was replaced every 24hrs. The number of viable cells was counted using trypan blue when controls were about 90% confluent. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment in presence of LY294002 or SH-5 led to significant growth inhibition (both $p < 0.002$), but no significant growth inhibition was found using $1\alpha,25(\text{OH})_2\text{D}_3$ treatment alone ($p = 0.4$) by ANOVA.

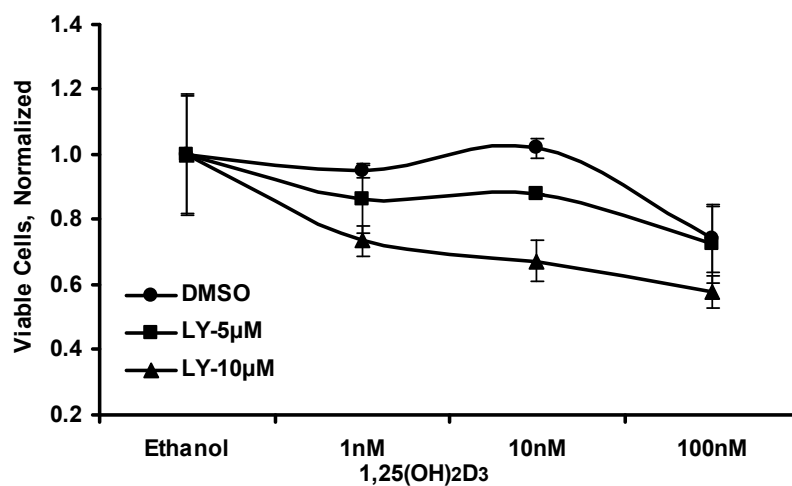


Figure 13. LY294002 and $1\alpha,25(\text{OH})_2\text{D}_3$ synergistically inhibit growth of tumor-derived $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitive *Pten null* MPEC. $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitive *Pten null* MPEC (clone 11) were grown and treated as described in the Materials and Methods section.

TABLE II

Synergism between 1 α ,25(OH) $_2$ D $_3$ and LY294002 in tumor-derived *Pten null* MPEC***

1,25(OH) $_2$ D $_3$, nM	LY294002, μ M	CI*	DRI**		Synergism
			1,25(OH) $_2$ D $_3$	LY294002	
10	10	0.220	4.9*10 4	4.6	Strong
100	10	0.133	8969.7	7.5	Strong

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism).

**Dose-reduction Index. DRI is calculated using the equation: $(DRI)_1=(D_x)_1/D_1$ and $(DRI)_2=(D_x)_2/D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination, and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone.

*** Data are from assays depicted in Figure 11; only mean values that are significantly different by ANOVA are depicted.

3.5. DISCUSSION

Recently, the process of senescence has received a lot of interest and has been suggested to play two significant roles in human cancer. First, induction of senescence has been shown to serve as a barrier for progression of tumorigenesis (125,143,144). Second, senescence has been shown to act as an acute, drug-inducible arrest mechanism that may contribute to positive outcomes from cancer therapy (88,92,105).

In the present study, we have demonstrated that acute loss of Pten expression in mouse prostatic epithelial cells leads to an increased number of senescent cells, as evidenced by enlarged morphology (Figures 8,10) and positive SA- β -galactosidase staining (Figures 7-10). This observation is consistent with the studies which have shown that activation of AKT1 or acute loss of *Pten* leads to induction of senescence in mouse prostate cells (111,112). In addition, it has been shown that suppression of the PI3K pathway in normal human diploid fibroblasts is sufficient to induce senescence (122). This finding was further supported by the observation that PI3K-deficient MEF cell lines cannot be established, as the cells become senescent in culture (145). Our observation that the number of senescent MPEC increases when Pten expression is lost further confirms the involvement of the Pten/Akt axis in senescence responses.

When we treated the Pten-expressing MPEC and MPEC with acute loss of Pten with $1\alpha,25(\text{OH})_2\text{D}_3$, we observed that, in parallel with initially higher senescence levels, $1\alpha,25(\text{OH})_2\text{D}_3$ treatment induced senescence in a significantly higher percentage of the MPEC with acutely-lost Pten compared to the Pten-expressing counterparts. This suggests that $1\alpha,25(\text{OH})_2\text{D}_3$ might act through the same molecular pathways implicated in Pten-loss-mediated senescence, or through another complimentary pathway. Importantly, the higher induction of senescence by $1\alpha,25(\text{OH})_2\text{D}_3$ in MPEC with acute loss of Pten expression was accompanied by a higher level of growth inhibition in these cells. For instance, 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ inhibited growth of 50% of

Pten-expressing cells, while the same dose inhibited growth of 99% of cells with Pten expression knocked down by shRNA (Figure 2).

It has previously been shown that in prostate, up-regulation of p27 and p53 is essential for the induction of senescence associated with AKT1 activation or Pten loss, respectively (111,112). On the other hand, the anti-proliferative actions of $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cells almost universally involve up-regulation of p21 and/or p27 (20,24,25). Thus, it can be speculated that the p53-p21/p27 axis might be implicated in the increased senescence and growth inhibition by $1\alpha,25(\text{OH})_2\text{D}_3$ in cells with acute loss of Pten expression. Our earlier studies demonstrated that induction of p21 is implicated in the induction of senescence by $1\alpha,25(\text{OH})_2\text{D}_3$ in a prostate cancer cell line (29), but the exact molecular mechanisms of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence in prostate cells remains to be elucidated, and is a focus of ongoing research in our laboratory.

Recently, the notion of using senescence as a mechanism to slow or stop cancer-prone cell from progressing to malignant disease has received much attention (146). Moreover, the potential of small molecules targeting regulators of senescence to induce acute senescence in oncogene-stimulated cells has been demonstrated. Three recent studies (all published in 2010) have shown that decreasing the activity of G1 CDKs or enhancing p53 expression might lower the ‘critical point’ for triggering senescence in oncogenically primed cells (124,128,129).

Lin et al. (128) used a mouse model of complete prostate Pten inactivation, in which the animals eventually develop prostate cancer. Skp2, a protein that mediates degradation of a number of proteins including p21 and p27 (147), was then genetically eliminated. The resulting Skp2 deficiency restricted prostate cancer development by triggering cellular senescence *in vivo*, leading to persistently smaller tumors with reduced invasiveness. Importantly, Lin et al. also demonstrated that a small-molecule drug that blocks the activity of Skp2 induces senescence in a PC3 prostate cancer cell line which is both Pten-null and p53-null, representing one of the most aggressive genetic states encountered in human cancer (128). The growth of PC3 tumors treated with this drug *in vivo* was also inhibited. Interestingly, coherent with these findings, Skp2

silencing in PC3 and DU145 cells, which have also evaded the p53 response, triggered cellular senescence and cooperated with the DNA-damaging agent doxorubicin to induce cellular senescence and growth arrest (128).

Campaner and colleagues have demonstrated that loss of Cdk2 causes sensitization to Myc-induced senescence in pancreatic β -cells or splenic B-cells *in vivo*, correlating with delayed lymphoma onset in the latter (129). Two small-molecule inhibitors of CDK2 were able to introduce senescence in Myc-overexpressing human leukemia cells, leading to a decrease in proliferation, but this effect was not seen when the inhibitors were used to treat cells expressing normal levels of Myc (129).

Alimonti and colleagues reported that administering Nutlin-3, a p53-stabilizing Mdm2 inhibitor (148), to Pten^{pc/-} mice prior to tumor onset led to reduction in both tumor size and the number of affected prostate glands. This was associated with an increase of β -gal activity and p53 and p21 staining in all the glands analyzed (124). Together, these studies suggest that it might be feasible to use pro-senescence therapies in oncogenically-primed mouse cells to reduce the tumorigenic potential of these cells.

The present study supports the hypothesis that $1\alpha,25(\text{OH})_2\text{D}_3$ (or its precursors) may be used as a pro-senescence therapeutic agent for the treatment of early neoplastic disease of prostate. This hypothesis is based on several observations: (i) our data that demonstrates the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to selectively inhibit growth and induce senescence in cells with acutely lost Pten (which by itself induces senescence), (ii) a high prevalence of loss of PTEN function in human prostate tumors (53-54), (iii) the observation that human PIN lesions are commonly associated with induction of senescence, which is absent in the normal tissues or in prostate cancer tissues (111,112), (iv) the observation that as many as 30% of men with a diagnosis of PIN on biopsy are found to harbor an invasive prostatic adenocarcinoma on repeat biopsy (130), and, (v) demonstration of the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to upregulate p21 and p27 levels (131), decrease Cdk2 activity (26) and downregulate Skp2 levels (24,26), all of which have been implicated in

oncogene-induced senescence. Thus, further studies evaluating the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to slow or prevent the cancer-prone prostate cells from progressing to malignant disease, as well as role of senescence in these processes, are highly warranted.

Use of $1\alpha,25(\text{OH})_2\text{D}_3$ to prevent progression of prostate neoplastic disease is further supported by a study by Banach-Petrosky et al. that showed that in the *Nkx3-1*; *Pten* mutant mice, an another putative model for prostate carcinogenesis, $1\alpha,25(\text{OH})_2\text{D}_3$ administration delayed the development of PIN, and $1\alpha,25(\text{OH})_2\text{D}_3$ demonstrated better antitumor activity when administrated to mice with early-stage (PIN) rather than advanced-stage prostate disease (149). Involvement of senescence in the protective $1\alpha,25(\text{OH})_2\text{D}_3$ effects, however, was not evaluated in this study.

Another possible alternative to $1\alpha,25(\text{OH})_2\text{D}_3$ use for treatment of early-stage prostate neoplastic disease is use of its precursor, $25(\text{OH})\text{D}_3$. It has been shown that prostate cells express 1α -hydroxylase, and thus can synthesize $1\alpha,25(\text{OH})_2\text{D}_3$ from $25(\text{OH})\text{D}_3$ locally (150-153). In addition, $25(\text{OH})\text{D}_3$ inhibits growth of prostate cells to a degree comparable with $1\alpha,25(\text{OH})_2\text{D}_3$ when tested in culture (150,151). Moreover, in humans, $25(\text{OH})\text{D}_3$ can be administered at significantly higher doses than $1\alpha,25(\text{OH})_2\text{D}_3$ without inducing hypercalcemic effects (154). All of the above suggest that $25(\text{OH})\text{D}_3$, the precursor of $1\alpha,25(\text{OH})_2\text{D}_3$, should be tested for its potential to exhibit responses similar to the ones we observed using $1\alpha,25(\text{OH})_2\text{D}_3$ including an ability to induce senescence in prostate cells.

Oncogene-induced senescence is one of the phases in the process of tumorigenesis. The oncogene-stimulated senescent cells eventually may acquire an ability to evade senescence by modulating the function or levels of proteins involved in the senescence response. Taking into consideration that $1\alpha,25(\text{OH})_2\text{D}_3$ may act on the same targets that are implicated in senescence induction, we sought to test whether $1\alpha,25(\text{OH})_2\text{D}_3$ retains its ability to inhibit growth of *Pten null* MPEC that have already progressed to invasive cancer. For that reason, we isolated MPEC from mouse prostate tumors where the tumorigenesis process was driven by homozygous deletion of

the *Pten* gene (we note that these animals were of the same genetic background as the animals used for isolation of *Pten*^{lox/lox} MPEC used for Cre-mediated *Pten* deletion *in vitro*). These tumor-derived *Pten null* MPEC represent later stages of disease progression. We observed that tumor-derived *Pten null* MPEC lost their sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition (Figure 11). We speculate that one of the later events in the tumorigenesis process rendered these cells insensitive to antiproliferative qualities of $1\alpha,25(\text{OH})_2\text{D}_3$. The $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitivity was partially rescued by combination of $1\alpha,25(\text{OH})_2\text{D}_3$ with an AKT or PI3K inhibitor, leading to a synergistic growth inhibition with the latter (Figure 12, Table II). Moreover, our previous data demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ with AKT inhibitors synergize to inhibit growth of prostate cancer cell lines DU145 (WT PTEN) and LNCaP (PTEN null) as well as primary prostate cancer cell strains (29). Synergistic growth inhibition by combination of $1\alpha,25(\text{OH})_2\text{D}_3$ and an AKT inhibitor, API-2, was associated with a cooperative G1 arrest as well as induction of senescence (29). Thus, in addition to its possible preventative role, $1\alpha,25(\text{OH})_2\text{D}_3$ might be beneficial for treatment of advanced prostate malignancy when combined with PI3K or AKT inhibitors.

Conventional chemotherapy and radiotherapy have been previously shown to induce cellular senescence in tumor samples of cancer patients, but at the cost of cytotoxic effects on normal and cancer cells (89), as well as an increased risk of secondary malignancies later in life (155,156). In this sense, targeted therapy oriented to modulate pro-senescence mediators could avoid the unselective genotoxicity delivered by conventional chemotherapeutics, thereby representing an improved therapeutic strategy. Our preliminary data supports the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to selectively inhibit growth of cells that have lost PTEN expression. The elucidation of the molecular and mechanistic details of this process is warranted, and studies on relevance to human disease would be worthwhile.

To inject a note of caution, several negative factors associated with senescence-inducing therapies *in vivo* have to be acknowledged. First, senescent cells *in vitro* have been shown to release several pro-inflammatory cytokines, chemokines and tissue-remodeling enzymes, which

have been suggested to act in a cell autonomous as well as a paracrine manner (120,121). While these modulators might be involved in the clearance of senescent cells by the immune system (101), they may also stimulate the malignant phenotype in nearby tumor cells (157).

Second, health and cellular growth rates in human prostate are heavily dependent on interaction of epithelial and stromal cells (158), thus possible effects associated with presence of senescent epithelial cells on epithelial-stromal interactions and overall organ health have to be evaluated in the future.

Third, it is conceivable that cancer cells in a senescent-like state might remain as “dormant” tumor cells, presenting a risk for tumor relapse. Although it was recently discovered that senescent cells can drive tumor clearance by stimulating the immune response (101), it is critical to obtain more knowledge on the mechanisms responsible for senescent tumor cell clearance. However, since prostate cancer is generally considered a slow-growing cancer, pro-senescence therapy might present less of a concern than for more rapidly growing cancers, especially for the treatment of men with early-stage, slow-growing tumors where a ‘watchful waiting’ approach is recommended.

In conclusion, we have demonstrated that acute *in vitro* loss of Pten leads to an increased sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ antiproliferative actions and senescence induction. This suggests that $1\alpha,25(\text{OH})_2\text{D}_3$ might have potential as a selective pro-senescent therapeutic for earlier stages of prostate cancer. This is especially relevant given the existing evidence suggesting that most elderly men have foci of latent neoplastic disease in the prostate (2,3) and often present with clinical vitamin D deficiency (159,160). We found that cells isolated from *Pten null* invasive tumors lost sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition, implying that additional events during the tumorigenesis process led to abrogation of $1\alpha,25(\text{OH})_2\text{D}_3$ -sensitivity in these cells. Nonetheless, $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitivity in these cells was partially rescued by combination with AKT or PI3K inhibitors, leading to a synergistic growth inhibition. Thus, in addition to a potential as a preventative pro-senescence therapeutic, $1\alpha,25(\text{OH})_2\text{D}_3$ in

combination with PI3K or AKT inhibitors may be beneficial in later stages of prostate cancer.

These findings lay a foundation for future studies to further evaluate the pro-senescence activities of $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs and their relevance to human disease.

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CHAPTER IV

GENERAL DISCUSSION

Importance and Future Directions

Our data demonstrated that treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ leads to significantly higher levels of growth inhibition and senescence induction in MPEC with *in vitro* acute deletion of Pten compared to their Pten-expressing counterparts. In contrast, tumor-derived *Pten null* MPEC showed a complete loss of sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition, which was partially restored by co-treatment with a PI3K or AKT inhibitor. A novel mechanism for the antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$, senescence, was discovered. We also demonstrated that when used in combination, $1,25(\text{OH})_2\text{D}_3$ and pharmacological inhibitors of AKT synergize to inhibit growth of prostate cancer cells and cooperate to induce G1 arrest, senescence and higher p21 protein levels. The potential importance of these observations as well as future directions for the research project are the subject of this section.

4.1. Synergistic Growth Inhibition by $1\alpha,25(\text{OH})_2\text{D}_3$ and PI3K/AKT Inhibitors

The use of AKT inhibitors is complicated by the importance of this pathway for the cellular physiology of multiple tissues and organs. Patients receiving AKT inhibition-based therapy can develop severe toxicities, at least temporarily. Depending on the type of AKT inhibitor, these toxicities may include insulin-resistance, infertility and neurotoxicity as well as exacerbation of preclinical glaucoma, Alzheimer's disease, Parkinson's disease and schizophrenia (1-4). While current trials are evaluating whether significant efficacy is achievable without excessive toxicity, it has also been suggested that AKT inhibitors may have greater utility as an adjuvant therapy with other agents rather than as a primary drug (5).

In the present study, we have demonstrated that combination treatment with an AKT inhibitor and $1\alpha,25(\text{OH})_2\text{D}_3$ leads to synergistic growth inhibition of prostate cancer cells. Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ or an AKT inhibitor alone can be associated with toxicities, but the combination of the two by allowing for synergistic anticancer activities may have the advantage of limiting the side-effects associated with either of the individual drugs, while providing additive and potentially synergistic therapeutic effects. This possibility warrants further evaluation in *in vivo* models and, if the outcome is positive, in human clinical trials. One important reason for exploring the therapeutic potential of this combination is that the observed synergism seems to be independent of the Pten status of the cells, as $1\alpha,25(\text{OH})_2\text{D}_3$ and PI3K or AKT inhibitors synergized to inhibit growth of PTEN null LNCaP and Pten null MPEC as well as in PTEN WT DU145 cells, and thus may offer a therapeutic option for late-stage prostate cancer, where few viable treatment options are available. Another, equally important reason to pursue further studies of this combination therapy is that the synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ and an AKT inhibitor was not restricted to prostate cancer cells, as similar effects were also observed in pancreatic cancer cell lines. This suggests that such combination therapy may also have relevance to other types of human cancers. Further evaluation of the therapeutic potential of the synergism between $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT inhibitors should be of special interest regarding the treatment of two frequently lethal cancers, glioblastoma and breast cancer, where AKT activation (through PTEN loss or otherwise) is commonly observed (6).

Another important feature of this drug combination is that the synergistic growth inhibition produced was associated with cooperative induction of senescence, which may be a valuable alternate route to tumor suppression when apoptosis has been disabled or repressed. AKT inhibitors are known to induce apoptosis in cancer cells when used at higher doses (7-9), but for our combination studies, we used AKT inhibitors at lower concentrations which did not cause apoptosis or pronounced growth inhibition if used alone. In prostate cancer cell line DU145 and in a human primary prostate cancer cell strain, the AKT inhibitor at such a concentration in

combination with $1\alpha,25(\text{OH})_2\text{D}_3$ produced senescence-associated morphology and SA- β -gal activity. Tumor cells that present with abnormal activation of AKT pathways, while being the primary target of AKT inhibitor-based therapy, are often resistant to the pro-apoptotic stimuli of conventional therapeutics (10,11). It can be speculated that ability of $1\alpha,25(\text{OH})_2\text{D}_3$ alone or in combination with other agents, such as AKT inhibitors, to induce senescence could provide an alternative for treatment of apoptosis-resistant tumors, and this possibility should be further investigated. However, the balance between apoptosis and senescence induction upon treatment may depend on the stage and nature of a tumor, the expression and activity of proteins involved in the cell cycle and apoptosis, and other intra-tumoral conditions. Thus, further understanding the molecular milieu responsible for this balance is needed in order to identify phenotypes and molecular signatures of tumors that would benefit from pro-senescent therapies.

We have found that cooperative induction of senescence by $1\alpha,25(\text{OH})_2\text{D}_3$ and inhibition of the AKT pathway in prostate cancer cells is paralleled by cooperative induction of p21, but not p27. The important role of p21 in induction of senescence as well as its ability to stimulate growth arrest are well known (12). It has been shown that tumor cells can be induced to undergo senescence through overexpression of p21 (13,14), and that, induction of senescence in tumor cells by various anticancer agents is also heavily dependent on p21 expression. In addition, p21 inhibition or knockout can decrease the induction of senescence by severalfold (15).

Some studies suggest that up-regulation of p21 in senescent cells, while critical, is often a transient event (16). Interestingly, the length and magnitude of p21 induction were shown to be important factors in determining the ability of cells to resume the normal cell cycle and form colonies after senescence induction (17). This phenomenon was studied in a model where tumor cells expressed p21 from an inducible promoter. In this model, the induction of p21 expression led to a senescent phenotype, but regardless of the duration or magnitude of p21 induction, after shutoff of p21 expression all cells initially reentered the cell-cycle and replicated their DNA (17). However, the ability of cells to form colonies after promoter shutoff was inversely correlated with

the duration and magnitude of p21 expression, with very few cells recovering after prolonged induction (4-5 days). But importantly, most of the cells reentering the cell-cycle died of mitotic catastrophe upon entering mitosis, or underwent senescence-like growth arrest in the subsequent cycle. p21 induction was found to inhibit the expression of multiple proteins involved in the execution and control of mitosis explaining the observed mitotic abnormalities (17). While we observed cooperative induction of p21 by combination of $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT inhibitors, the duration of this upregulation and the overall importance of p21 upregulation for the senescence phenotype induced by this drug combination were not assessed and remain to be elucidated.

The next steps for this study should include evaluation of the effects of combinational treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT inhibitors in an *in vivo* model, as well as the determination of *critical* molecular players involved in the interaction of the two compounds. In addition, our preliminary data demonstrates that not only $1\alpha,25(\text{OH})_2\text{D}_3$, but also $25(\text{OH})\text{D}_3$ in combination with an AKT inhibitor, API2, synergistically inhibits growth of prostate cells (data not shown). Bearing in mind that $25(\text{OH})\text{D}_3$ can be administered at significantly higher doses than $1\alpha,25(\text{OH})_2\text{D}_3$ without inducing hypercalcemic effects (18), this particular combination could have promise and should also be evaluated. Another logical step is to test the combination of $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT inhibitors in treating cancers where risk of development has been shown to be strongly dependent on vitamin D status, such as colon and breast cancer.

4.2. Pros and Cons of Senescence-inducing Therapies

The majority of current anti-tumor strategies are designed to induce an apoptotic response in the tumor cells. The use of this strategy is limited due to the high toxicity of apoptosis-inducing drugs to normal tissues, and the pro-survival alterations present in or acquired by cancer cells, which often inhibit apoptosis. It has recently been suggested that senescence-inducing drugs, by attacking tumor cells from a different angle, might present an effective therapeutic approach for

treatment of cancer either alone or in combination with classic apoptosis-promoting therapies, and that such senescence-inducing drugs may be less toxic to normal tissues.

Selectivity. Acutely induced senescence can affect the growth of tumor cells while sparing the healthy surrounding tissues, and this ability is supported by substantial laboratory evidence. In mouse models of both spontaneous and radiation-induced tumors where p53 can be switched on and off, most of the sarcomas, lymphomas and liver carcinomas that developed in the absence of p53 regressed after p53 restoration, which promotes senescence (19,20). Interestingly, sarcomas and liver carcinomas regressed in association with a potent induction of senescence which was observed only in the tumor cells, leaving normal tissues completely unaffected (19). Another study showed that use of pro-senescent CDK2 inhibitors induced senescence and inhibited growth of the cells expressing oncogenic Myc, while having no effect on growth or senescence induction in cells without Myc overexpression (21). Similarly, a p53-stabilizing drug induced senescence and inhibited growth only in *Pten*^{-/-} MEFs, while having no significant effect on Pten WT MEFs (22).

Our studies have demonstrated that 1 α ,25(OH)₂D₃ inhibits growth and induces senescence in cells with lost Pten expression to a much higher extent than in their Pten-expressing counterparts. This observation suggests that 1 α ,25(OH)₂D₃ could be a useful pro-senescent therapeutic, selectively targeting oncogene-stimulated cells while sparing the healthy adjacent cells - at least in the context of PTEN loss, which is a very common event in human prostate cancer. Interestingly, there is also other evidence that 1 α ,25(OH)₂D₃'s antiproliferative qualities are selective for cancer-prone cells. It has been shown that 1 α ,25(OH)₂D₃ induces apoptosis and cell cycle arrest in tumor-derived endothelial cells (TDECs), while not significantly affecting growth of endothelial cells isolated from normal tissues (23,24).

Secretory Phenotype. Studies have demonstrated that senescent cells *in vitro* release several pro-inflammatory cytokines, chemokines and tissue-remodeling enzymes, which affect the cells in an autocrine as well as a paracrine manner (25,26). Some studies suggest that these secreted factors can stimulate the growth and angiogenic potential of nearby premalignant cells (27-31). In prostate cancer cells, however, it was recently found that while the presence of senescent cancer cells increases the proliferation of co-cultured cells *in vitro*, *in vivo* senescent cancer cells failed to increase the establishment, growth or proliferation of LNCaP or DU145 xenografts in nude mice (32).

Cells in which senescence was stimulated by oncogene expression were also shown to have a so-called secretory phenotype (26). Indeed, the evaluation of expression profiles of cells undergoing senescence has revealed that oncogene activation is intrinsically associated with the induction of a pro-inflammatory program *in vitro* (33) as well as *in vivo* (34). However, it was recently demonstrated that some of these secreted factors play a causative and necessary role in the establishment and maintenance of the senescent condition. For instance, interleukins 6 (IL-6) and 8 (IL-8) and their receptors were necessary for the induction of senescence in human cells (25,26). Inactivation of these interleukins and some of the pathways they affect prevented entry into senescence or allowed escape from it. Moreover, factors secreted by senescent cells might be involved in the clearance of senescent cells by the immune system, leading to reduced tumor growth (20). Together, these data encourage further efforts to develop senescence induction therapies, although more studies confirming the absence of negative effects of the secretory products of senescent cells are certainly needed. Such studies may be particularly important in considering potential senescence-inducing therapies for prostate cancer, because a complex interaction of stromal and epithelial cells is heavily implicated in the regulation of prostate epithelial cell growth, and commonly occurs via secreted proteins acting in both paracrine and autocrine manner (35).

Senescence as an Alternative Treatment Outcome in Apoptosis-resistant Tumors. Given the rapid induction of apoptosis and the rather delayed induction of senescence in response to anticancer therapy *in vivo*, it has been suggested that the senescence machinery may act as a back-up program to substitute for or to reinforce an insufficient apoptotic response (36).

Some observations in radiation therapy suggest that the induction of permanent cytostatic arrest could be a later treatment response in certain clinical cases. In particular, complete regression of prostate cancers was reported to take more than a year in some patients (37), and regression of desmoids tumors took up to two years after radiation treatment (38). This slow course of tumor disappearance seems consistent with radiation-induced senescence. It has been speculated that intrinsically chemo-sensitive cells such as hematological cancer cells may use senescence as secondary mechanism, while typically less apoptosis-susceptible solid tumors, including prostate tumors, might respond to chemotherapy by drug-induced senescence as a primary mechanism (39).

Recent studies have shown that cancer cells carrying certain mutations are resistant to drug-induced apoptosis, but such drugs are still able to induce a senescence response. For instance, treatment with cyclophosphamide is unable to induce apoptosis in Myc-induced tumors that overexpress BCL2, but such treatment is still capable of inducing cytostasis and senescence (40). In the same study, ARF-deficient lymphomas that showed apoptotic defects were still capable of entering senescence after chemotherapy (40). This suggests the potential value of using a senescence induction treatment for the treatment of certain types of apoptosis-resistant cells.

On the other hand, some cell types (but not all) might become resistant to certain apoptosis stimuli once they acquire the senescent phenotype. For example, senescent human fibroblasts are more resistant to ceramide-induced apoptosis than their younger counterparts (41). But this effect is not uniform, and the type of apoptotic stimuli and the molecular signature of the senescent cell seem to determine the outcome of the treatment. For instance, senescent human fibroblasts that

are resistant to apoptosis caused by growth factor deprivation and oxidative stress are still sensitive to apoptosis stimulated through Fas death receptor (42,43).

Together, these data demonstrated that drug-induced senescence might slow proliferation of cancer cells that are resistant to apoptosis; however, in some cases senescent cells might acquire resistance to certain types of pro-apoptotic stimuli. These observations raise a note of caution. First, it is possible that in certain cases pro-senescent therapies applied in earlier stages of tumorigenesis may lead to an increased resistance to pro-apoptotic therapies later. Second, a careful consideration has to be given to the order of administration of pro-apoptotic and pro-senescence drugs in combinational therapies. Preliminarily, it can be speculated that pro-apoptotic agents should be administered before pro-senescence agents to allow cancer cells with intact apoptosis machinery to be killed by the pro-apoptotic drug, leaving apoptosis-resistant cells (with intact senescence machinery) to be forced into senescence by administration of a senescence-inducing agent. Before pro-senescence compounds alone or in combination with pro-apoptotic agents can be safely used, more studies evaluating the long-term effects of pro-senescence therapies will need to be carried out. Cell type, stage of tumorigenesis and expression of proteins involved in apoptosis and senescence induction in a cancer cell may determine whether a pro-apoptotic or pro-senescence approach is an appropriate approach for treatment, and when in the course of the disease.

Considering these questions in the case of $1\alpha,25(\text{OH})_2\text{D}_3$, our data suggest that $1\alpha,25(\text{OH})_2\text{D}_3$ might be more effective in earlier stages of prostate cancer, particularly in neoplasms driven by loss of PTEN function and associated with senescence induction. The obvious limitation of our study is the use of a mouse cell culture model, which might not be representative of a human prostate with its complex organization and control of cell growth. However, we believe that our results provide a foundation for further investigation of $1\alpha,25(\text{OH})_2\text{D}_3$ as a pro-senescence therapeutic, which studies should be carried in models more relevant to human disease.

Clearance of Senescent Cells. While there are examples of senescent cells residing for years in the organism (44), recent studies suggest that cells that have undergone oncogene-induced senescence can be engulfed by phagocytic cells in a process of that is essentially similar to the removal of apoptotic cells (20). In these studies, tumor regression associated with senescence induction was accompanied by the presence of tumor-infiltrating neutrophils, macrophages and natural killer cells (20). Further supporting the notion that senescent cells are phagocytized, it was shown that senescent neutrophils, like apoptotic granulocytes (45), might ultimately face phagocytosis through a yet unknown recognition mechanism (46). In addition to phagocytosis, it was suggested that senescent cells may eventually eliminate themselves by autophagy (47). However, scenarios when senescent cells reside in the tissue or organ for a long time are associated with potential risks such as re-initiation of cell division or adverse effects on organ/tissue function, which are discussed below.

Risk of Re-initiation of Cell Division. Re-initiation of cell division in senescent cells is still possible under some circumstances (48). *In vitro* analysis has demonstrated that senescent cells re-enter the cell-cycle upon acquisition of additional senescence-compromising mutations leading to loss of p53, p16 or pRb (48-50). Likewise, MCF-7 breast cancer cell clones that escaped from adriamycin-induced senescence were found to express higher than parental levels of CDC2/CDK1 kinase (51).

In an *in vivo* model, drug-inducible senescence improved outcomes from cancer therapy in the Myc-transgenic mouse model, but virtually all mice harboring drug-induced senescent tumor cells eventually succumbed to the neoplastic disease, due to selection of cells with loss of expression of proteins that are essential for senescence (40). On the other hand, a study by Chang and colleagues showed that cells that were forced into senescence by temporary overexpression of p21, while they were able to reenter the cycle and replicate their DNA, died through mitotic

catastrophe in the same cycle or underwent senescence-like growth arrest in a subsequent cell cycle (17).

Senescence and Aging. While cellular senescence was proposed to be an anti-cancer or tumor-suppressive mechanism protecting organisms from cancer, it also represents the aging process, or loss of regenerative capacity of cells *in vivo* (reviewed in (52)). Senescent cells are found in many renewable tissues including the haematopoietic system, vasculature, stroma and many epithelial organs, and their numbers, while relatively rare in young organisms, increases with age (48,53,54). The contribution of senescence to aging has been explained by the principle of antagonistic pleiotropy (52). That is, when properly controlled, senescence has positive functions, for example in wound healing or tumor suppression, while uncontrolled senescence can lead to a chronic accumulation effect eventually impairing functionality of tissues and organs.

4.3. Potential of $1\alpha,25(\text{OH})_2\text{D}_3$ Use as a Pro-senescence Therapeutic in Prostate Cancer

Evidence suggests that most elderly men have foci of latent neoplastic disease in the prostate (55,56). Many of these prostate tumors grow so slowly that they may never threaten a patient's life, thus, there is a danger of overtreatment. This is a particularly important issue since treatment for prostate cancer is often associated with significant side-effects. Thus, low-morbidity pro-senescence therapeutics that slow disease progression provide an attractive opportunity for treatment of patients with early-stage neoplastic prostate disease, or for prophylactic treatment in groups with high-risk for prostate cancer development.

We have demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits growth and induces senescence in prostate cells with lost PTEN expression to a higher degree than their PTEN-expressing counterparts. It was previously shown that acute loss of Pten leads to PIN formation (57), and experimental data supports the beneficial use of pro-senescent therapeutics to slow the process of tumorigenesis (58). We therefore hypothesize that $1\alpha,25(\text{OH})_2\text{D}_3$ (or its precursor $25(\text{OH})\text{D}_3$) may be a good

candidate for use in treatment of early-stage prostate neoplastic disease, especially disease associated with PTEN loss. We speculate that men with benign tumors of prostate or men with slow-growing cancer who defer active treatment (i.e. men undergoing expectant management or “watchful waiting”) may be ideal candidates for low-morbidity $1\alpha,25(\text{OH})_2\text{D}_3$ -based interventional therapies.

In addition to use of $1\alpha,25(\text{OH})_2\text{D}_3$ (or its precursor $25(\text{OH})\text{D}_3$) for slowing growth of earlier stages of prostate tumor formation, our results demonstrate that it can be used for treatment of advanced prostate cancer in combination with PI3K or AKT inhibitors. This might be particularly relevant to prostate cancers that are known to be resistant to pro-apoptotic conventional therapies, as $1\alpha,25(\text{OH})_2\text{D}_3$ in combination with AKT inhibitors may rely on induction of senescence to slow or inhibit tumor growth.

Specifically to prostate neoplasms, pro-senescence therapy utilizing $1\alpha,25(\text{OH})_2\text{D}_3$ alone or in combination with other therapeutic agents (possibly AKT inhibitors) presents less of a risk for adverse effects normally associated with pro-senescence therapies. First, possible re-initiation of cell division of cells with drug-induced senescence presents less of a concern due to the overall slow growth rate of prostate tumors. Second, potential adverse effects associated with presence of senescent cells in prostate tissue is not expected to critically affect the health of the patient due to the lower fundamental importance of this organ, especially at older ages. While our *in vitro* data suggest a potential role for $1\alpha,25(\text{OH})_2\text{D}_3$ for treatment of prostate neoplasms, whether it indeed presents an effective therapeutic allowing for slowing the growth of pre-cancerous and/or cancerous prostate tumors *in vivo* remains to be established, and will be tested in our laboratory in the near future. Future studies should also evaluate the molecular mechanisms underlying $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence, as well as potential players involved in $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence, which may refine patient selection for $1\alpha,25(\text{OH})_2\text{D}_3$ -based interventions. The less calcemic form of vitamin D, $25(\text{OH})\text{D}_3$, as well as synthetic vitamin D analogs may also present an interesting subject matter for future studies.

In conclusion, our findings suggest that $1\alpha,25(\text{OH})_2\text{D}_3$ (or its precursor, $25(\text{OH})\text{D}_3$) might be a good candidate to be used for treatment of early stages of prostate tumorigenesis associated with loss of PTEN and/or induction of senescence, while its utilization in combination with PI3K or AKT inhibitors might be useful for treatment of advanced prostate cancers, and these possibilities should be further explored (Figure 1).

4.4. Potential Players in $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated Senescence

Our results have demonstrated that in cells with acute loss of Pten expression, which are already showing signs of senescence, $1\alpha,25(\text{OH})_2\text{D}_3$ was able to further induce senescence, and, importantly, to a significantly higher extent than in comparable Pten-expressing cells. While the molecular players in this selective induction of senescence in cells with lost Pten expression by $1\alpha,25(\text{OH})_2\text{D}_3$ remain to be identified, several potential players can be suggested based on the experimental data already in the literature.

The Potential Role of p53-p21 Axis. While PTEN has been shown to protect p53 from Mdm2-mediated degradation (59), it can be speculated that loss of Pten would lead to lower levels of p53. However, the opposite results were obtained in a study by Chen et al., where prostate-specific deletion of Pten led to increased levels of p53 and p53-dependent senescence (57). They showed that *Pten*^{-/-} prostate cells isolated from *Pten*^{-/-} prostates contained more than 10-fold more p53 protein than the *Pten* Wt cells, in turn leading to a p53-dependent accumulation of p21 (57).

At the same time, regulatory pathways related to the p53 gene are implicated in $1\alpha,25(\text{OH})_2\text{D}_3$'s antiproliferative signaling. VDR is a transcriptional target of p53 (60), and it was demonstrated that p53 protein binds to conserved intronic sequences of the VDR gene *in vivo* and induces its expression as well as expression of VDR target genes, in a vitamin D₃-dependent manner (60). Thus, it can be speculated that Pten-loss mediated induction of p53 may lead to

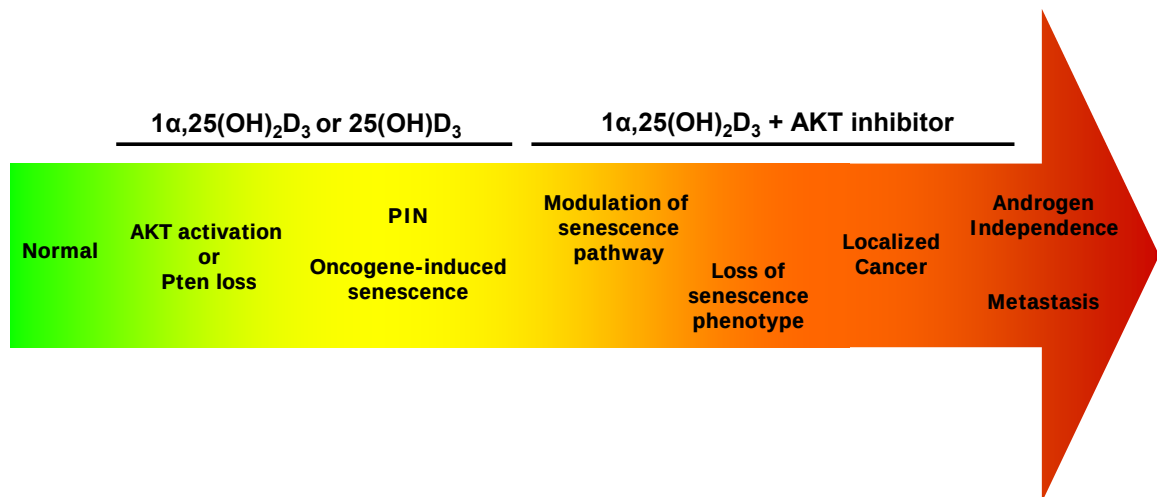


Figure 1. Suggested use of $1,25(\text{OH})_2\text{D}_3$ for treatment of prostate neoplasms and cancer.

increased expression of VDR, the levels of which are playing a critical role in the antiproliferative signaling of $1\alpha,25(\text{OH})_2\text{D}_3$ (61). These higher levels of VDR, upon binding to its ligand, $1\alpha,25(\text{OH})_2\text{D}_3$, can induce transcription of the target genes stimulating $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition.

There is another indirect suggestion of a critical role of p53 in VDR signaling. While cells transformed with human papilloma virus (HPV), which is known to target Rb proteins (62), are not resistant to $1\alpha,25(\text{OH})_2\text{D}_3$'s antiproliferative effects; cells transformed with simian virus 40 (SV40), which leads to suppression of the transcriptional properties of the p53 and pRb (63), are resistant to $1\alpha,25(\text{OH})_2\text{D}_3$'s antiproliferative effects (64,65). These results were demonstrated in prostate cancer cell lines (64) as well as in breast cancer cell lines (65). In the latter study, the authors showed that expression of the large T antigen of SV40 strongly inhibited $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated VDRE transcriptional activity, and increasing the VDR concentration reversed the inhibitory effect of the large T antigen and restored the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition (65). Together these data further suggest that the p53 was essential for the growth inhibitory potential of $1\alpha,25(\text{OH})_2\text{D}_3$ in these cells. In LNCaP cells, however, while p53 was not required to induce growth inhibition by $1\alpha,25(\text{OH})_2\text{D}_3$, elimination of its function allowed the cells to recover from the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth arrest (66), suggesting that $1\alpha,25(\text{OH})_2\text{D}_3$ may exhibit p53-dependent as well as p53-independent anticancer effects in LNCaP cells.

A downstream target of p53, CDK inhibitor $\text{p}21^{\text{Kip1/Cip1}}$, has for the last decade appeared to be the most promising target for $1\alpha,25(\text{OH})_2\text{D}_3$'s cell growth regulatory effects (67). p21 has been shown to be a critical target for $1\alpha,25(\text{OH})_2\text{D}_3$'s antiproliferative effects in multiple cell types, including prostate cells (68). For instance, stable transfection of prostate cancer cells ALVA-21 with a p21 antisense construct abolishes $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition (69,70). The human $\text{p}21^{\text{Waf1/Cip1}}$ gene is a primary $1\alpha,25(\text{OH})_2\text{D}_3$ -responding gene, with at least three functional VDR binding promoter regions (71), in two of which VDR and p53 were shown to co-localize

(72). Therefore, p53 may play role in $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition by regulating VDR-mediated transcription of p21.

Evaluation of p53 and p21 protein levels and their localization in Pten expressing MPEC and in MPEC that have lost Pten expression before and after treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ may shed light on the involvement of p53 and p21 in differential ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to induce growth inhibition and senescence in these cells. One approach to determine the importance of either of the proteins would be targeted deletion followed by evaluation of response to $1\alpha,25(\text{OH})_2\text{D}_3$ treatment.

The Potential Role of p27. A study by Majumder and colleagues showed that prostate-specific deletion of Pten or expression of Myr-AKT1 led to up-regulation of p27 levels (73). This finding was quite surprising, since it had been generally accepted that loss of PTEN or activation of AKT is associated with downregulation of $p27^{\text{Kip1}}$, as shown in a number of *in vitro* systems (74-82). Majumder et al. (73) demonstrated that this p27 induction was essential for the induction of senescence in the Pten loss-driven PIN. Importantly, human PIN was also shown to be associated with upregulated levels of p27 as well as markers of senescence, suggesting a relevance of these observations to human disease.

And yet, $1\alpha,25(\text{OH})_2\text{D}_3$ is known to up-regulate levels of p27 (83-86). This up-regulation occurs indirectly, but rather through increased stability of p27 mediated by inhibition of p27's degradation. While the essential role of p21 for $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition is well-established (66,70), the role of p27 up-regulation is less clear. Our laboratory has shown that $p27^{\text{Kip1}}$ is essential for the antiproliferative action of $1\alpha,25(\text{OH})_2\text{D}_3$ in primary, but not immortalized MEFs (87). Another group found that while induced by $1\alpha,25(\text{OH})_2\text{D}_3$, p27 was dispensable for $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition (88). Nonetheless, given the critical role of p27 in Pten loss-mediated senescence induction and PIN formation, evaluation of p27

levels as well as their importance for the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence warrant further study.

$1\alpha,25(\text{OH})_2\text{D}_3$ -mediated induction of p27 is dependent on the localization and activity of proteins involved in its degradation. Briefly, p27's up-regulation by $1\alpha,25(\text{OH})_2\text{D}_3$ was shown to be a result of an $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated decrease in Cdk2 activity (89) or down-regulation of Skp2 levels (90), both of which play a role in degradation of p27. Interestingly, down-regulation of Cdk2 and Skp2 have been shown to play a critical role in oncogene-induced senescence as well (91). Thus, down-regulation of Cdk2 and Skp2 may be implicated in the mechanism for $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence as well and is discussed below.

The Potential Role of Cdk2. Cyclin-dependent kinase 2 (CDK2) is a member of the cyclin-dependent kinase family that regulates G₁ to S phase progression of the cell cycle (92). The role of Cdk2 in senescence was recently demonstrated by Campaner and colleagues, who reported that loss of *Cdk2* increased senescence in E μ -Myc-expressing pancreatic and splenic B-cells *in vivo*, correlating with delayed lymphoma onset in the latter (21). Further, a pharmacological inhibitor of Cdk2 induced growth inhibition and senescence in E μ -Myc MEFs while not affecting growth or senescence of WT MEFs (21).

Yet, CDK2 activity can be regulated by $1\alpha,25(\text{OH})_2\text{D}_3$ (88,89). $1\alpha,25(\text{OH})_2\text{D}_3$ induces nuclear to cytoplasmic relocation of CDK2, which was shown to be essential for the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated G₁ arrest and growth inhibition of prostate cancer cells (88). Together, these data suggest that evaluation of $1\alpha,25(\text{OH})_2\text{D}_3$'s effects on CDK2 levels, activity and localization may provide more insights into mechanisms of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence induction.

The Potential Role of SKP2. SKP2 is a critical component of the SKP2-SCF complex, which acts as an E3 ligase to target p27 and other substrates for ubiquitylation and degradation

(93,94). Recent studies suggest that SKP2 may have oncogenic activity, as SKP2 overexpression is frequently observed in human cancers (95,96). Conversely, reduction of SKP2 activity may play a role in inducing senescence. Very recently it has been shown that Skp2 deficiency profoundly enhances cellular senescence upon Pten inactivation in prostate (91). Moreover, a pharmacological inhibitor of the SKP2-SCF complex triggered senescence in PC3 prostate cancer cells, both *in vitro* and *in vivo* (91).

Similarly, $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to negatively regulate SKP2 expression and activity, which may also be related to inducing senescence. $1\alpha,25(\text{OH})_2\text{D}_3$ and its analog EB1089 were capable of reducing SKP2 protein levels (89), which can occur through inhibition of SKP2 promoter activity in a VDR-dependent manner (90). In addition to reduction of SKP2 protein levels, $1\alpha,25(\text{OH})_2\text{D}_3$ (and its analog EB1089) were shown to inhibit association of SKP2 with p27, leading to decreased degradation of p27 in the cells (84). Taken together, these data suggest a potential role of SKP2 in $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated senescence.

4.5. Additional Benefits of Restoration of Adequate Vitamin D Levels

Vitamin D metabolism starts to change around mid-life: cutaneous production of vitamin D declines and renal function begins to deteriorate. Conversion of 7-dehydrocholesterol to previtamin D_3 in human skin exposed to UVB radiation is decreased as much as twofold in elderly subjects (97). This diminished ability to produce vitamin D in the skin of older subjects is often aggravated by changes in life-style and other environmental factors. In addition, chronic use of sunscreen, which is commonly recommended to be used to prevent skin cancer, can dramatically reduce serum vitamin D levels (98). Therefore, elderly are commonly at risk of becoming vitamin D deficient (99).

It is well established that adequate vitamin D levels are important for calcium homeostasis and bone health. In addition, low vitamin D levels are correlated with higher risk of myocardial

infraction (100,101), type I diabetes (102,103), multiple sclerosis (104,105), and falls (106), all of which further support the benefits of vitamin D supplementation.

An interesting observation was made recently: diagnosis of breast (107), colon (108) and prostate cancer (109,110) is the highest in the winter months. In addition, diagnosis during months in which vitamin D levels would be expected to be the lowest (winter/spring) as well as low vitamin D levels at the time of cancer diagnosis have been reported to be an indicator of poor prognosis in lung, colorectal, and breast cancer (111,112). Therefore, it is currently recommended to promptly restore the usually deficient serum 25(OH)D₃ level of all individuals with newly diagnosed cancers, unless hypercalcemia is present (113).

In addition, treating advanced prostate cancer patients with vitamin D metabolites might improve their quality of life. A small study of men with metastatic AIPC were treated with 2000 IU 25(OH)D₃ daily, and analysis of the results of the study demonstrated an improvement in bone pain scores and an improvement in muscle strength measurements compared to placebo control (114). Beer and colleagues also reported significant analgesic activity of 1 α ,25(OH)₂D₃ when combined with docetaxel treatment in men with metastatic AIPC (115).

Together, these data show that administration of vitamin D compounds, aside from the therapeutic effects, would provide additional health benefits, especially in elderly men who are commonly vitamin D-deficient.

4.6. Conclusions

In the present study we have discovered a novel mechanism of 1 α ,25(OH)₂D₃-mediated growth inhibition – senescence. We have demonstrated that 1 α ,25(OH)₂D₃ treatment induced higher levels of growth inhibition and expression of senescence markers in cells with acute *in vitro* loss of Pten compared to their Pten-expressing counterparts. MPEC isolated from *Pten null* tumors had no growth inhibitory response to 1 α ,25(OH)₂D₃, suggesting that additional mutation(s) that occur during the process of carcinogenesis can lead to loss of 1 α ,25(OH)₂D₃-

sensitivity in these cells. The growth inhibitory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ was partially rescued by combination with AKT or PI3K inhibitors, leading to a synergistic growth inhibition with the latter. $1\alpha,25(\text{OH})_2\text{D}_3$ and AKT or PI3K inhibitors synergistically inhibited growth of prostate cancer cells and cooperated to induce cell cycle arrest and senescence.

These results, if further confirmed by *in vivo* as well as by clinical studies, may lead to the use of therapeutic doses of $1\alpha,25(\text{OH})_2\text{D}_3$ (or its precursor $25(\text{OH})\text{D}_3$) for: (i) slowing the tumorigenesis process in early-stage prostate neoplastic disease by inducing cellular senescence, especially in cells that lose PTEN function; and/or (ii) treatment of more advanced prostate cancer in combination AKT inhibitors (Figure 1).

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APPENDIX

MATERIALS AND METHODS

Materials. $1\alpha,25(\text{OH})_2\text{D}_3$ (Biomol, Plymouth Meeting, PA) was reconstituted in 100% ethanol and stored at -80°C . LY294002 (Sigma-Aldrich Co., St Louis, MO) and API-2 (Calbiochem, La Jolla, CA) were reconstituted in DMSO and stored at -20°C .

Tissue culture. Panc-1 and BXPC-3 pancreatic cancer cell lines (generously provided Dr. Gregory Kuchera (WFU)) were grown in RPMI-1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Growth Assays for Synergism Determination. Cells were plated at 10^4 cells per 35 mm dish in triplicate. To determine synergism cells were treated with increasing doses of API-2, increasing doses of $1\alpha,25(\text{OH})_2\text{D}_3$ or multiple combinations of API-2 and $1,25(\text{OH})_2\text{D}_3$. Briefly, 48 hr after plating the cell growth medium was replaced with 1 ml of experimental medium containing twice (2x) the indicated concentration of API-2 or vehicle (DMSO, $1x = 0.1\% \text{ V/V}$). One hour later 1 ml of medium containing twice the final concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ or vehicle (ethanol, $1x = 0.1\% \text{ V/V}$) was added to each dish. Cells remained in the experimental medium until the vehicle control cells reached 80-90% confluence, typically 5-7 days. Experimental medium was replaced every 48hr. Viable cells were counted with a hemacytometer after trypan blue exclusion.

Statistical Analyses. Synergism was assessed with CalcuSyn software (Biosoft, Ferguson, MO). Briefly, the dose effect for each drug alone was determined based on the experimental observations using the median effect principle: the combination index (CI) for each combination was calculated according to the following equation: $\text{CI} = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [(D)_1(D)_2/(D_x)_1(D_x)_2]$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect

when used alone. $CI = 1$ represents the conservation isobologram and indicates additive effects. CI values < 1 indicate a more than expected additive effect (synergism). Dose Reduction Index (DRI) for each drug and dose was calculated using the equation: $(DRI)_1 = (D_x)_1 / D_1$ and $(DRI)_2 = (D_x)_2 / D_2$. Statistical analyses for synergism experiments were performed using the statistical software package NCSS 2002 (Number Cruncher Statistical Systems, Kaysville, UT). Differences in growth data were determined by two-way ANOVA controlling for $1\alpha,25(OH)_2D_3$ or API-2 dose with post hoc analysis by Fisher's test. Cell cycle distribution and senescence analyses were performed using two-way ANOVA with post hoc analysis by Fisher's LSD test. In all cases, $P \leq 0.05$ was considered significant.

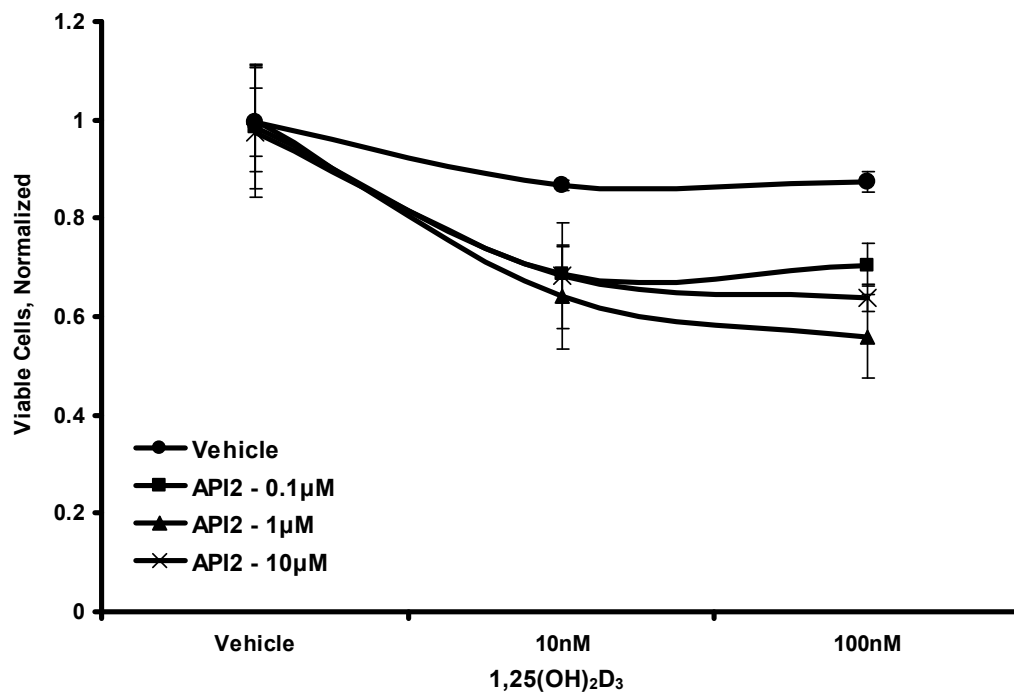


Figure AP-1. API-2 and 1,25(OH)₂D₃ synergistically inhibits growth of Panc-1 pancreatic cancer cells. Growth inhibition of Panc-1 in response to API-2 and 1,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in the Materials and Methods section. Each point represents the mean $\pm$ SD of triplicate plates after normalization of cell number to controls (0.1% ethanol).

TABLE AP-I

Synergism between 1,25(OH)₂D₃ and API-2 in Panc-1 pancreatic cancer cells***

1,25(OH) ₂ D ₃ , nM	API2, μ M	CI*	DRI**		Synergism
			1,25(OH) ₂ D ₃	API2	
10	0.1	0.042	2.0*10 ²⁹	23.6	Very Strong
10	1	0.018	2.4*10 ³⁷	54.3	Very Strong
10	10	0.086	2.0*10 ³⁹	11.6	Very Strong
100	0.1	0.054	4.9*10 ²⁷	18.6	Very Strong
100	1	0.007	5.3*10 ³⁸	135.6	Very Strong
100	10	0.053	3.6*10 ³⁹	18.8	Very Strong

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism).

**Dose-reduction Index. DRI is calculated using the equation: $(DRI)_1 = (D_x)_1 / D_1$ and $(DRI)_2 = (D_x)_2 / D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. DRI values for 1 α ,25(OH)₂D₃ treatment are high due to inability of the program to correctly calculate DRI based on the formula as the x effect of the 1 α ,25(OH)₂D₃ alone is near zero.

*** Data are from assays depicted in Figure AP-1, only statistically significantly different means by ANOVA are depicted.

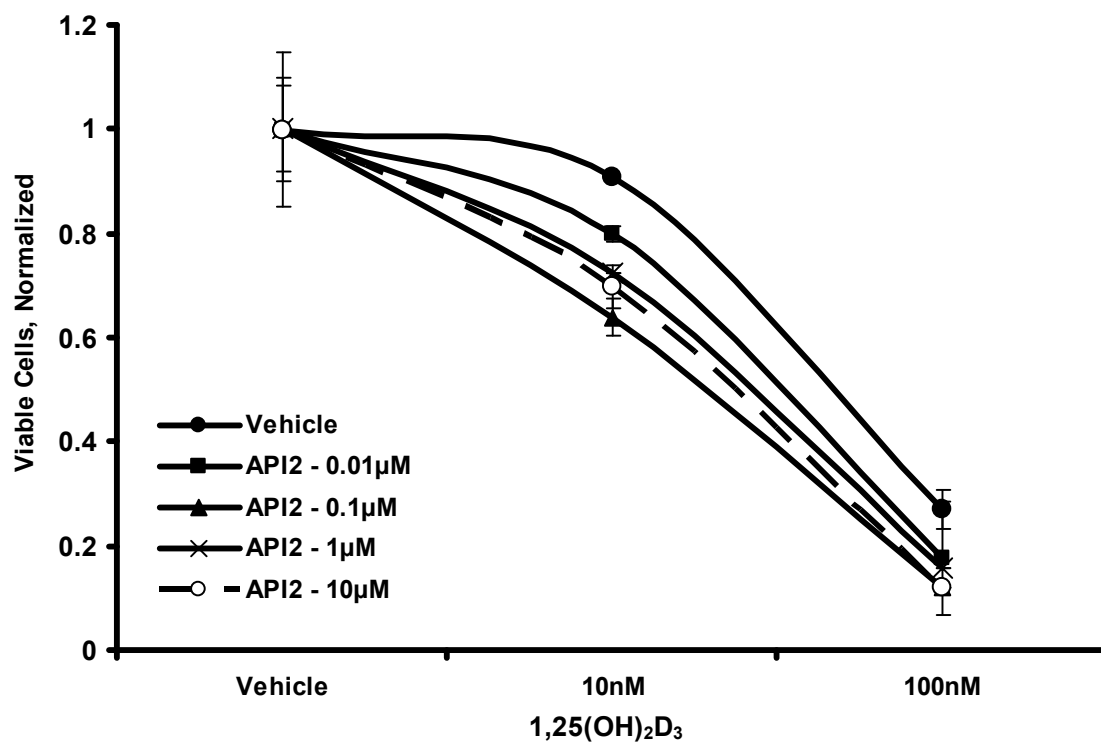


Figure AP-2. API-2 and 1,25(OH)₂D₃ synergistically inhibits growth of BXPC-3 pancreatic cancer cells. Growth inhibition of BXPC-3 in response to API-2 and 1,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in the Materials and Methods section. Each point represents the mean $\pm$ SD of triplicate plates after normalization of cell number to controls (0.1% ethanol).

TABLE AP-II

Synergism between 1,25(OH)₂D₃ and API-2 in BXPC-3 pancreatic cancer cells***

1,25(OH) ₂ D ₃ , nM	API2, μ M	CI*	DRI**		Synergism
			1,25(OH) ₂ D ₃	API2	
10	0.01	0.706	1.4	126.2	Moderate
10	0.1	0.335	3.1	70.1	Intermediate
10	1	0.155	7.5	47.7	Strong
10	10	0.353	7.3	4.6	Intermediate
100	0.1	0.142	7.0	6.7*10 ⁴	Strong
100	1	0.110	9.1	1.1*10 ⁴	Strong
100	10	0.0.82	12.3	2291.7	Very Strong

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism).

**Dose-reduction Index. DRI is calculated using the equation: $(DRI)_1 = (D_x)_1 / D_1$ and $(DRI)_2 = (D_x)_2 / D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone.

*** Data are from assays depicted in [Figure AP-2](#), only statistically significantly different means by ANOVA are depicted.

SCHOLASTIC VITA

LINARA AXANOVA

EDUCATION

2004 – present	pursuing Ph.D., Cancer Biology Department, Wake Forest University
2002-2004	M.S., Foods and Nutrition, Foods and Nutrition Department, Purdue University
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SCHOLASTIC AND PROFESSIONAL EXPERIENCE

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2002-2004	Research Assistant, Foods and Nutrition Department, Purdue University
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Feb-Dec. 2001	Visiting Researcher, Department of Agricultural and Biological Engineering, Purdue University
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AWARDS/HONORS/GRANTS

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PUBLICATIONS

Axanova, L., Chen, Y., McCoy T., Sui G. and Cramer, S. 1,25-dihydroxyvitamin D₃ and PI3K/AKT Inhibitors Synergistically Inhibit Growth and Induce Senescence in Prostate Cancer Cells. *Prostate* (E-publication ahead of print), 2010

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ABSTRACT AND PRESENTATION

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