Published in final edited form as:
Cancer Res. 2013 April 1; 73(7): 2289–2297. doi:10.1158/0008-5472.CAN-12-4119.

Inhibition of protein kinase CK2 reduces *CYP24A1* expression and enhances 1,25-dihydroxyvitamin D_3 anti-tumor activity in human prostate cancer cells

Wei Luo[1], **Wei-Dong Yu**[1], **Yingyu Ma**[1], **Mikhail Chernov**[2], **Donald L. Trump**[3], and **Candace S. Johnson**[1,*]

[1]Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York

[2]Department of Small Molecule Screening Core, Roswell Park Cancer Institute, Buffalo, New York

[3]Department of Medicine, Roswell Park Cancer Institute, Buffalo, New York

Abstract

Vitamin D has broad range of physiological functions and anti-tumor effects. 24-hydroxylase, encoded by the *CYP24A1* gene, is the key enzyme for degrading many forms of vitamin D including the most active form, $1,25D_3$. Inhibition of *CYP24A1* enhances $1,25D_3$ anti-tumor activity. In order to isolate regulators of *CYP24A1* expression in prostate cancer cells, we established a stable prostate cancer cell line PC3 with *CYP24A1* promoter driving luciferase expression to screen a small molecular library for compounds that inhibit *CYP24A1* promoter activity. From this screening, we identified, 4,5,6,7-tetrabromobenzimidazole (TBBz), a protein kinase CK2 selective inhibitor as a disruptor of *CYP24A1* promoter activity. We show that TBBz inhibits *CYP24A1* promoter activity induced by $1,25D_3$ in prostate cancer cells. In addition, TBBz downregulates endogenous *CYP24A1* mRNA level in TBBz treated PC3 cells. Furthermore, siRNA-mediated CK2 knockdown reduces $1,25D_3$ induced *CYP24A1* mRNA expression in PC3 cells. These results suggest that CK2 contributes to $1,25D_3$ mediated target gene expression. Lastly, inhibition of CK2 by TBBz or CK2 siRNA significantly enhanced $1,25D_3$ mediated anti-proliferative effect *in vitro* and *in vivo* in a xenograft model. In summary, our findings reveal that protein kinase CK2 is involved in the regulation of *CYP24A1* expression by $1,25D_3$ and CK2 inhibitor enhances $1,25D_3$ mediated anti-tumor effect.

Keywords

1,25-dihydroxyvitamin D_3; *CYP24A1*; protein kinase CK2; prostate cancer

Introduction

The most physiologically active form of the prohormone, vitamin D_3 (cholecalciferol), is 1,25-dihydroxyvitamin D_3 ($1,25D_3$). $1,25D_3$ plays a key role in the regulation of calcium homeostasis and bone metabolism through effects on tissues such as bone, gut and kidney (1, 2). Non-classical roles for $1,25D_3$ including the regulation of proliferation, differentiation and immune function have now been identified in a variety of cell types (3). The serum level

*Corresponding author: Candace S. Johnson, Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, Phone: 716-845-8300, Fax: 716-845-1258, Candace.Johnson@roswellpark.org.

of $1,25D_3$ is highly regulated through synthesis facilitated by 1-alpha-hydroxylase (*CYP27B1*), and through inactivation by 24-hydroxylase (*CYP24A1*) (1, 2).

CYP24A1 is transcriptionally regulated by the interaction between the vitamin D receptor (VDR)-retinoid-X-receptor (RXR) heterodimer and vitamin D response elements (VDREs) on *CYP24A1* gene (4–6). In the absence of $1,25D_3$, VDR/RXR hetero-dimers bind to these VDREs and repress transcription through interactions with a co-repressor complex that has histone de-acetylase activity (7). In the presence of $1,25D_3$, the co-repressor complex is released, permitting the recruitment of a co-activator complex that leads to the activation of the gene (8, 9). $1,25D_3$ also stimulates rapid non-genomic effects in some cell-types via the ERK1/ERK2/ERK5, PKC, or JNK MAP kinase modules through a cell-membrane-associated VDR (3).

High *CYP24A1* expression level is a common feature of several solid tumors (3, 10–15) and is associated with poorer prognosis (10, 14, 16). The increased intra-tumoral levels of *CYP24A1* would lead to rapid degradation of $1,25D_3$, thus, limiting the amount of $1,25D_3$ locally in the tumor cells and abrogating the anti-proliferative, or pro-differentiation effects of $1,25D_3$ (10, 16, 17). Inhibition of *CYP24A1* is expected to slow the catabolism of $1,25D_3$, thereby enhancing the anti-proliferative effect of $1,25D_3$ (18–21). Administration of $1,25D_3$ in combination with a *CYP24A1* inhibitor enhances the anti-tumor activity of $1,25D_3$ (19, 22). However, most of the current *CYP24A1* inhibitors, such as ketoconazole, are relatively non-specific, and strikingly increase the *CYP24A1* expression level compared to cells treated with $1,25D_3$ alone (19).

In the present study, we screened a small molecule library to identify novel *CYP24A1* inhibitors using a *CYP24A1* promoter-driving luciferase reporter assay. Furthermore, we expected that the new *CYP24A1* inhibitor would enhance $1,25D_3$-mediated function by inhibiting *CYP24A1* expression.

Materials and methods

Materials

$1,25D_3$ was purchased from Tetrionics (Madison, WI). $25D_3$, LOPAC^{1280} and 4,5,6,7-tetrabromobenzimidazole (TBBz) were obtained from Sigma-Aldrich (St. Louis, MO). The dual-luciferase assay kit was supplied by Promega (Madison, WI). Mouse anti-CYP24 antibody was a gift from Cytochroma Inc. (Markham, Ontario, Canada). Anti-CK2α (H-286, sc-9030) antibody and anti-actin antibody were from Santa Cruz biotechnology (Santa Cruz, CA). Anti-cleaved Caspase-3 (Asp175, #9661) antibody was purchased from Cell Signaling Technology (Danvers, MA). Anti-Ki-67 antibody was purchased from Leica Microsystems (NCL-Ki67p; Buffalo Grove, IL). TaqMan® Gene Expression Assay for *CYP24A1* (Hs00167999_m1), *CSNK2A1* (Hs00751002_s1), *CDKN1A* (Hs00355782_m1), Growth arrest and DNA-damage-inducible protein 45a *(GADD45A*, Hs00169255_m1) and the transient receptor potential vanilloid type 6 gene (*TRPV6,* Hs00367960_m1) were purchased from Applied Biosystems (Foster City, CA). ON-TARGET plus SMARTpool siRNA specific for human *CSNK2A1* (CK2α1, L-003475), ON-TARGET plus Non-targeting Pool (D-001810), and DharmaFECT 2 transfection reagents were purchased from Dharmacon (Thermo Fisher Scientific Dharmacon, Lafayette, CO). Human RNA from 30 paired human prostate normal and primary tumor lesions were obtained from Department of Pathology, Roswell Park Cancer Institute and approved by Institutional review board.

Cell lines

The prostate cancer cell lines DU145 and PC3 were purchased from American Type Culture Collection (ATCC) and used within 6 months after resuscitation. Cell lines were

authenticated by ATCC with short tandem repeat (STR) DNA profiling and cytogenetic analysis. Cells were maintained in culture according to providers' protocols for a maximum of 10 passages (one month).

Generation of stable reporter cell line

pGL4.21 vector expressing the firefly luciferase gene under the control of *CYP24A1* promoter was constructed by the insertion of *CYP24A1* promoter using NheI and XhoI restriction enzyme sites (23). A stable human prostate cancer PC3 cell line expressing *CYP24A1* promoter-driving luciferase reporter (PC3/*CYP24A1*) was generated by transfection using lipofectamine 2000 followed by puromycin selection.

Chemical library and high throughput screening

Screening was performed by Small Molecule Screening Core Facility (SMSC) at the Roswell Park Cancer Institute using LOPAC^{1280} library. PC3/*CYP24A1* cells were seeded to 96-well plate (10^4/well) overnight. 120 nL of each compound or DMSO was added to the plate for 20 minutes using a JANUS robotic liquid handler (PerkinElmer) equipped with 96-pinn tool (V&P Scientific), followed by the addition of 1,25D$_3$ to a final concentration of 100 nM. The final concentration of the library compounds in the media was 10 μM. After 24-h incubation, luciferase activity for each well was assayed using SteadyGlo kit (Promega) and luminescence measured using Envision multilabel plate reader (PerkinElmer). Hits were defined as over 50% inhibition of 1,25D$_3$ mediated *CYP24A1* promoter-driving luciferase reporter activity.

CK2 small interfering RNA (siRNA)

PC3 cells were plated in 6-well plates (10^5/well) overnight. Cells were transfected with 50 nM siRNA-CK2 or Non-targeting siRNA for 72 h using Dharma-FECT 2 transfection reagent following the manufacturer's instruction. Following transfection, the cells were treated with vehicle EtOH or 1,25D$_3$ for 6 h or 48 h and harvested for experiments as indicated.

Quantitative reverse transcriptase PCR (qRT-PCR)

Expression of *CK2*, *CYP24A1, TRPV6*, *p21^{Waf1}* and *GADD45A* mRNA was assessed by qRT-PCR using TaqMan® Gene Expression Assay and normalized to the human *GAPDH* and samples were analyzed in triplicate.

Immunoblotting analysis

Whole cell lysates were prepared and Western blot analysis performed as described previously (24).

Trypan blue exclusion assay

PC3 cells or PC3 cells transfected with siRNA-CK2 were plated in 6-well plates (3×10^4/well) for 24 h and treated with 5 μM of TBBz or/and 100 nM of 1,25D$_3$ or 1000 nM of 25D$_3$. Cells were trypsinized and viable cell count measured using ViCell XR (Beckman Coulter) on day 3, 6 and 9.

Tumor growth assay

PC3 cells (2×10^6) were inoculated subcutaneously into the right flank of male SCID mice (6–8 weeks old). At day 8–9 post implantation, when the tumors were palpable (6.5 × 5 mm), animals were treated with 1,25D$_3$ (15.5 μg/kg/d × 3, i.p. weekly), TBBz three times weekly (15 mg/kg/d, i.p., every 2 days), or the combination for 2 weeks. Body weight was

monitored twice a week. Tumor growth was assessed and calculated as described previously (22, 25). The mice protocols used in tumor growth assay were approved by the Institutional Animal Care and Use Committee at Roswell Park Cancer Institute.

Immunohistochemistry

Tissue staining with anti-Ki-67 and anti-cleaved Caspase-3 was conducted as described previously (22).

TUNEL assay

Nuclear DNA fragmentation in situ was detected using TACS-XL In Situ Apoptosis Detection Kit according to the manufacture's instruction (R&D system, Minneapolis, MN).

Statistics

Statistical significance of data was determined by two-tailed Student's *t* test. Wilcoxon Signed-Rank test was performed to compare the expression levels of *CK2* in paired normal and tumor samples. 2×2 contingency table was performed to analyze the correlation between increased *CYP24A1* expression and *CK2* expression in paired normal and tumor human prostate samples.

Results

Screening of small molecules from LOPAC^{1280} Library by *CYP24A1* promoter driving reporter assay

A stable human prostate cancer PC3 cell line expressing *CYP24A1* promoter-driving luciferase reporter was generated by transfection using lipofectamine 2000 followed by puromycin selection. Screening of the LOPAC^{1280} library in this system resulted in the identification of 70 hits each of which had over 50% inhibition of $1,25D_3$-induced *CYP24A1* promoter activity. (Fig. 1A) Excluding the hits with high toxicity, known from Small Molecule Screening Core (SMSC) database, twenty-one selected molecules were subjected to secondary dose-response experiments to confirm initial observations. Seventeen hits reduced $1,25D_3$-mediated *CYP24A1* promoter activation (Fig. 1B). Among them, 4,5,6,7-tetrabromobenzimidazole (TBBz) displayed the strongest inhibitory effect and was chosen for further investigation (Fig. 1A and 1B).

Repression of *CYP24A1* transcriptional activity by TBBz

To confirm the results from the screening, we examined the effect of various concentration of TBBz on *CYP24A1* promoter activity in PC3/CYP24A1 cells. Results showed that TBBz inhibited *CYP24A1* promoter activity in a dose dependent manner (Fig. 1C). We also tested the effect of TBBz on endogenous and $1,25D_3$-regulated *CYP24A1* expression. PC3 cells were treated with 1 or 5 μM of TBBz alone or followed by 10 nM, 30 nM and 100 nM of $1,25D_3$. qRT-PCR results showed that PC3 cells displayed low endogenous *CYP24A1* mRNA level and dose-dependent induction of *CYP24A1* mRNA expression by $1,25D_3$ (Fig. 1D and Supplementary Fig. 1A). TBBz significantly ($P < 0.01$) reduced $1,25D_3$-induced *CYP24A1* mRNA expression in a dose-dependent manner (Fig. 1D). Less induction of *CYP24A1* expression by lower dose of $1,25D_3$ was relatively less influenced by TBBz (Supplementary Fig. 1A). These results indicate that TBBz inhibits endogenous and $1,25D_3$-induced *CYP24A1* expression at the transcriptional level. Furthermore, we observed that $25D_3$, the precursor to $1,25D_3$ also induced *CYP24A1* mRNA expression in PC3 cells and TBBz inhibits $25D_3$-induced *CYP24A1* expression at the transcriptional level in PC3 cells (Supplementary Fig. 2A).

Reduction of *CYP24A1* expression by siRNA-CK2

TBBz is a selective protein kinase CK2 inhibitor (26). To investigate whether CK2 plays a role in the regulation of *CYP24A1* expression, siRNA-CK2 was transfected in prostate cancer PC3 or DU145 cells for 72 hours followed by the addition of $1,25D_3$. *CYP24A1* mRNA and *CYP24A1* protein were measured by qRT-PCR and Western blot, respectively. Results showed that PC3 and DU145 cells express CK2 and CK2 was effectively knocked down by siRNA-CK2 in PC3 and DU145 cells compared to control siRNA (Fig. 2A and 2B). $1,25D_3$ significantly ($P < 0.01$) increased *CYP24A1* expression in PC3 cells, which express a low level of endogenous *CYP24A1* (Fig. 2A and 2C, respectively), and in DU145 cells, which display a high level of endogenous *CYP24A1* (Fig. 2B and 2D, respectively). Transfection with siRNA-CK2 significantly reduced $1,25D_3$-induced *CYP24A1* expression at both mRNA and protein level in PC3 (Fig. 2A and 2C) and DU145 cells (Fig. 2B and 2D) as compared with the mock-transfected or siRNA control transfected samples. These results indicate that CK2 plays a role in regulation of $1,25D_3$-induced *CYP24A1* expression.

Differential effects of siRNA-CK2 on 1,25D3-induced *TYPV6*, $p21^{Waf1}$ and *GADD45A* mRNA expression

To investigate whether silencing of CK2 affects the expression of other vitamin D target genes, *TRPV6*, $p21^{Waf1}$ and *GADD45A* mRNA expression was measured by qRT-PCR in CK2 knockdown PC3 cells. $1,25D_3$ induced *TRPV6*, $p21^{Waf1}$ and *GADD45A* mRNA expression. siRNA-CK2 significantly ($P < 0.01$) reduced $1,25D_3$-induced *TRPV6* mRNA expression as compared to the mock-transfected or siRNA control transfected cells (Fig. 3A). In contrast, knockdown of CK2 markedly increased $p21^{Waf1}$ and slightly increased *GADD45A* mRNA expression (Fig. 3B and 3C). These results indicate that silencing of CK2 differentially affects the expression of vitamin D target genes.

Correlation of increased *CYP24A1* expression with increased CK2 expression in human prostate tumors

We analyzed mRNA expression of *CYP24A1* and *CK2* in 30 matched pair of human normal and tumor prostate samples by qRT-PCR. *CK2* expression was significantly increased in prostate tumor lesions compared to normal lesions ($P = 0.0224$) (Fig. 4). There was no correlation of the level of *CYP24A1* expression and *CK2* expression with Gleason Score. To determine whether tumor samples with increased *CYP24A1* expression correlated with increased *CK2* expression compared to normal samples, we built a 2×2 contingency table by dividing the 30 samples based on the *CYP24A1* expression change (≥ 1.5 fold up *vs.* other) and *CK2* expression change (≥ 1.5 fold up *vs.* other). Seven samples display increased *CYP24A1* expression in a total of 30 prostate tumors compared to matched normal prostate samples. Four of the 7 samples with increased *CYP24A1* expression have increased *CK2* expression. However, among the remaining 23 samples with low *CYP24A1* expression, only 3 samples have high *CK2* expression (Table 1). Fisher's exact test shows that increased *CYP24A1* expression is significantly associated with increased *CK2* expression in tumor (*P=0.0331*). These data indicate that *CK2* may be involved in regulation of increased *CYP24A1* expression in prostate cancer.

Enhancement of 1,25D3 anti-proliferative activity by TBBz or siRNA-CK2

To ascertain the potential therapeutic role of CK2 inhibitors in $1,25D_3$ anti-tumor action, cell viability and cell proliferation was examined using the trypan blue exclusion assay after treatment with $1,25D_3$, TBBz, or the combination of $1,25D_3$ and TBBz for 9 days. Combination treatment of $1,25D_3$ and TBBz resulted in a significant ($P < 0.05$) enhancement of $1,25D_3$ anti-proliferative effect in PC3 cells (Fig. 5A). We also measured *CYP24A1* mRNA expression on day 1 and day 9. We observed that *CYP24A1* mRNA

expression kept lower in PC3 cells treated with the combination of $1,25D_3$ and TBBz which showed the most antiproliferative activity compared to cells treated with $1,25D_3$ alone (Supplementary Fig. 1B). Instead of $1,25D_3$, $25D_3$ in combination of TBBz also caused a greater inhibition of proliferation in PC3 cells than when treated with either agent alone (Supplementary Fig. 2B).

To more specifically investigate the importance of CK2 in $1,25D_3$ anti-proliferative effect, siRNA-CK2 was employed. siRNA-CK2 significantly ($P < 0.05$) enhanced $1,25D_3$ anti-proliferative effect in PC3 cells (Fig. 5B). This indicates that CK2 inhibition is anti-proliferative and enhances $1,25D_3$ anti-proliferative effect.

Enhancement of 1,25D3 anti-tumor activity by TBBz *in vivo*

Having demonstrated the efficacy of the combination treatment of $1,25D_3$ and TBBz *in vitro*, we next assessed both toxicity and efficacy of the combination treatment in a PC3 prostate tumor xenograft mouse model. We observed a marked inhibition of tumor growth by the combination of $1,25D_3$ and TBBz, compared to $1,25D_3$ or TBBz alone (Fig. 6A). Mice grew normally without suffering from weight loss at a dose sufficient to induce anti-tumor effect (Fig. 6B). These results indicate that CK2 inhibitor TBBz enhances $1,25D_3$ anti-tumor activity *in vivo*.

Effect of the combination of 1,25D3 and TBBz on tumor cell *CYP24A1* expression, proliferation and apoptosis in *in vivo*

To investigate the effect of TBBz on *CYP24A1* expression *in vivo*, tumor tissues were harvested at the end of the treatment described in Fig. 5A, and *CYP24A1* mRNA expression was measured by qRT-PCR. $1,25D_3$ increased *CYP24A1* expression and TBBz reduced *CYP24A1* expression in tumors as compared to saline group (Fig. 6C). Furthermore, TBBz significantly ($P < 0.05$) reduced $1,25D_3$-induced *CYP24A1* expression (Fig. 6C). These observations were consistent with the results obtained in the *in vitro* study.

To further elucidate the molecular mechanisms for the anti-tumor activity of $1,25D_3$ and TBBz *in vivo*, we examined the proliferation marker Ki-67 and apoptosis marker cleaved caspase-3 as well as in situ DNA fragmentation (TUNEL) in tumor tissues (Fig. 6D). The results showed that saline-treated tumor tissue had strong Ki-67 staining, $1,25D_3$ or TBBz reduced Ki-67 staining (Fig. 6D). The combination of $1,25D_3$ and TBBz further reduced Ki-67 staining (Fig. 6D). We also observed that saline group did not have positive cleaved caspase-3 staining, whereas $1,25D_3$ or TBBz treatment alone induced caspase-3 cleavage in the tumor tissue (Fig. 6D). The combination of $1,25D_3$ and TBBz further enhanced caspase-3 cleavage (Fig. 6D). The effect of $1,25D_3$ and TBBz on apoptosis was further confirmed by TUNEL assay (Fig. 6D). These results clearly indicate the potential usefulness of the combination of $1,25D_3$ and CK2 inhibitors in prostate cancer therapy.

Discussion

The majority of *CYP24A1* inhibitors developed so far target the enzyme activity. However, decreased enzyme activity with current *CYP24A1* inhibitors is often associated with increased enzyme expression which negatively impacts on the vitamin D-mediated anti-tumor activity (19, 21, 27). In this study, the strategy we utilized to identify new *CYP24A1* inhibitors differs from previously described (28). We established a stable PC3 cell line, which express luciferase driven by *CYP24A1* promoter, to screen a small molecular library containing 1280 compounds. We identified 17 new *CYP24A1* inhibitors, TBBz being the strongest was selected for further characterization, which revealed a new *CYP24A1* expression regulating molecule, protein kinase CK2. Analysis of 30 paired normal and

tumor human prostate samples showed that increased *CYP24A1* expression is related to increased *CK2* expression in tumor. Moreover, we observed a significant enhancement of $1,25D_3$ anti-tumor activity by inhibiting CK2 *in vitro* or *in vivo*. The effects were associated with the reduction of *CYP24A1* expression, inhibition of proliferation and the induction of apoptosis in tumors.

Protein kinase CK2 is an evolutionarily conserved serine/threonine kinase which is ubiquitously expressed in human tissues. CK2 is located both in cytosol and nucleus (29, 30). Overexpression of CK2 has been noted in a variety of human cancers including prostate cancer and correlates with a poor clinical outcome (31–34). Inhibition of CK2 activity reduced cell proliferation in prostate cancer cells (35, 36). The role for CK2 in the regulation of *CYP24A1* gene expression in tumor has not been described before. Our study shows that CK2 positively regulates *CYP24A1* expression. We further show that CK2 expression was higher in tumor lesions compared to normal lesions ($P = 0.0224$) (Fig. 4). Increase of CK2 expression was significantly (*P=0.0331*) associated with increased CYP24A1 expression in these prostate tumor samples. These observations suggest that CK2 may serve as a mechanism for controlling *CYP24A1* expression in human cancers, and therefore supporting the use of CK2 inhibitors for cancer treatment in combination with $1,25D_3$.

CYP24A1 expression is heterogeneous in prostate cancer (23). It is noteworthy that three human prostate tumor samples with high *CK2* expression did not express high level of *CYP24A1* and three prostate tumor samples with high *CYP24A1* expression did not express high level of *CK2* compared to normal lesions (table 1). These data suggest that the level of *CK2* expression does not entirely account for the level of *CYP24A1* expression in human prostate tumor. Previous studies indicated multiple events are associated with *CYP24A1* expression in cancer, such as methylation and histone modification associated with the *CYP24A1* promoter (3, 23, 37), amplification at the CYP24A1 locus (13) and miRNA regulation (38).

We also observed that the silencing of CK2 differentially affects vitamin D target genes. We observed the significant reduction of $1,25D_3$-induced *TRPV6* in siRNA-CK2 transfected PC3 cells. Up-regulation of *TRPV6* by $1,25D_3$ in prostate cancer cells is considered to be pro-proliferative by increasing Ca^{2+}-uptake (39, 40). On the other hand, siRNA-CK2 enhanced $1,25D_3$-mediated induction of $p21^{Waf1}$ and *GADD45A*. $p21^{Waf1}$ is accounted in part for the anti-proliferative effects of VDR ligands on some cell types, such as prostate cancer (19, 41–43). *GADD45A* is identified as a primary target gene for $1,25D_3$ in ovarian, testicular and prostate cancer cells (19, 44, 45). The increase in *GADD45A* expression leads to a decrease of cyclin B and induces G2/M cell cycle arrest (45, 46). In the present study, the reduction of *CYP24A1* and *TRPV6* expression and the increase in the $p21^{waf1}$ and *GADD45A* expression by the combination of $1,25D_3$ and siRNA-CK2 may be reflective of the co-operative growth inhibition observed from the cell-proliferation assay.

However, the exact mechanisms underlying the effect of CK2 on $1,25D_3$-mediated *CYP24A1* induction remain unclear despite reports of CK2-mediated phosphorylation of purified VDR at $serine^{208}$ and VDRE construct transactivation in COS-7 kidney cells co-transfected with VDR and CK2 (47–49). Studies have shown that phosphorylation of hVDR at $serine^{208}$ does not affect the ability of VDR to bind to DNA and is not obligatory for $1,25D_3$ action, but may contribute to the modulation of the affinity of VDR for the vitamin D interacting protein (DRIP) complex, therefore increasing its ability to transactivate target promoters (50). At present, we cannot rule out additional mechanisms in the interaction between CK2 and vitamin D target genes as differential effect was observed on *TRPV6,* $p21^{Waf1}$ and *GADD45A*.

In summary, we developed a new strategy to identify novel *CYP24A1* inhibitors. Furthermore, we found that protein kinase CK2 is involved in the regulation of *CYP24A1* and other vitamin D target genes. CK2 inhibitor TBBz significantly enhances $1,25D_3$ anti-tumor activity *in vitro* and *in vivo*. These findings provide support for the combination treatment of CK2 inhibitor and vitamin D in prostate cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This study was supported by NIH/NCI grants CA067267, CA085142 and CA095045.

We thank Dr. Adam Karpf and Dr. Elizabeth A Griffiths for helpful discussions, Yan Li for statistical assistance, Mrs. Rui-Xian Kong for her excellent technical assistance, and Ms. Ellen Karasik for her excellent technical assistance in immunohistochemistry study.

References

1. Morris HA. Vitamin D: a hormone for all seasons--how much is enough? Clin Biochem Rev. 2005; 26:21–32. [PubMed: 16278774]
2. Omdahl JL, Morris HA, May BK. Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu Rev Nutr. 2002; 22:139–66. [PubMed: 12055341]
3. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nature reviews Cancer. 2007; 7:684–700.
4. Vaisanen S, Dunlop TW, Sinkkonen L, Frank C, Carlberg C. Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1alpha,25-Dihydroxyvitamin D3. J Mol Biol. 2005; 350:65–77. [PubMed: 15919092]
5. Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). Endocrinology and metabolism clinics of North America. 2010; 39:255–69. table of contents. [PubMed: 20511050]
6. Thompson PD, Jurutka PW, Haussler CA, Whitfield GK, Haussler MR. Heterodimeric DNA binding by the vitamin D receptor and retinoid X receptors is enhanced by 1,25-dihydroxyvitamin D3 and inhibited by 9-cis-retinoic acid. Evidence for allosteric receptor interactions. J Biol Chem. 1998; 273:8483–91. [PubMed: 9525962]
7. Dwivedi PP, Muscat GE, Bailey PJ, Omdahl JL, May BK. Repression of basal transcription by vitamin D receptor: evidence for interaction of unliganded vitamin D receptor with two receptor interaction domains in RIP13delta1. J Mol Endocrinol. 1998; 20:327–35. [PubMed: 9687155]
8. Jurutka PW, Whitfield GK, Hsieh JC, Thompson PD, Haussler CA, Haussler MR. Molecular nature of the vitamin D receptor and its role in regulation of gene expression. Rev Endocr Metab Disord. 2001; 2:203–16. [PubMed: 11705326]
9. MacDonald PN, Baudino TA, Tokumaru H, Dowd DR, Zhang C. Vitamin D receptor and nuclear receptor coactivators: crucial interactions in vitamin D-mediated transcription. Steroids. 2001; 66:171–6. [PubMed: 11179724]
10. Anderson MG, Nakane M, Ruan X, Kroeger PE, Wu-Wong JR. Expression of VDR and CYP24A1 mRNA in human tumors. Cancer Chemother Pharmacol. 2006; 57:234–40. [PubMed: 16180015]
11. Bareis P, Bises G, Bischof MG, Cross HS, Peterlik M. 25-hydroxy-vitamin d metabolism in human colon cancer cells during tumor progression. Biochem Biophys Res Commun. 2001; 285:1012–7. [PubMed: 11467853]
12. Friedrich M, Rafi L, Mitschele T, Tilgen W, Schmidt W, Reichrath J. Analysis of the vitamin D system in cervical carcinomas, breast cancer and ovarian cancer. Recent Results Cancer Res. 2003; 164:239–46. [PubMed: 12899526]

13. Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nature genetics. 2000; 25:144–6. [PubMed: 10835626]
14. Mimori K, Tanaka Y, Yoshinaga K, Masuda T, Yamashita K, Okamoto M, et al. Clinical significance of the overexpression of the candidate oncogene CYP24 in esophageal cancer. Ann Oncol. 2004; 15:236–41. [PubMed: 14760115]
15. Horvath HC, Lakatos P, Kosa JP, Bacsi K, Borka K, Bises G, et al. The candidate oncogene CYP24A1: A potential biomarker for colorectal tumorigenesis. The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society. 2010; 58:277–85. [PubMed: 19901270]
16. Chen G, Kim SH, King AN, Zhao L, Simpson RU, Christensen PJ, et al. CYP24A1 is an independent prognostic marker of survival in patients with lung adenocarcinoma. Clin Cancer Res. 2011; 17:817–26. [PubMed: 21169243]
17. Miller GJ, Stapleton GE, Hedlund TE, Moffat KA. Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1alpha,25-dihydroxyvitamin D3 in seven human prostatic carcinoma cell lines. Clin Cancer Res. 1995; 1:997–1003. [PubMed: 9816072]
18. Schuster I, Egger H, Herzig G, Reddy GS, Schmid JA, Schussler M, et al. Selective inhibitors of vitamin D metabolism--new concepts and perspectives. Anticancer Res. 2006; 26:2653–68. [PubMed: 16886676]
19. Yee SW, Campbell MJ, Simons C. Inhibition of Vitamin D3 metabolism enhances VDR signalling in androgen-independent prostate cancer cells. J Steroid Biochem Mol Biol. 2006; 98:228–35. [PubMed: 16483768]
20. Parise RA, Egorin MJ, Kanterewicz B, Taimi M, Petkovich M, Lew AM, et al. CYP24, the enzyme that catabolizes the antiproliferative agent vitamin D, is increased in lung cancer. Int J Cancer. 2006; 119:1819–28. [PubMed: 16708384]
21. Zhang Q, Kanterewicz B, Buch S, Petkovich M, Parise R, Beumer J, et al. CYP24 inhibition preserves 1alpha,25-dihydroxyvitamin D(3) anti-proliferative signaling in lung cancer cells. Mol Cell Endocrinol. 2012; 355:153–61. [PubMed: 22386975]
22. Muindi JR, Yu WD, Ma Y, Engler KL, Kong RX, Trump DL, et al. CYP24A1 inhibition enhances the antitumor activity of calcitriol. Endocrinology. 2010; 151:4301–12. [PubMed: 20591973]
23. Luo W, Karpf AR, Deeb KK, Muindi JR, Morrison CD, Johnson CS, et al. Epigenetic regulation of vitamin D 24-hydroxylase/CYP24A1 in human prostate cancer. Cancer Res. 2010; 70:5953–62. [PubMed: 20587525]
24. Ma Y, Yu WD, Kong RX, Trump DL, Johnson CS. Role of nongenomic activation of phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase 1/2 pathways in 1,25D3-mediated apoptosis in squamous cell carcinoma cells. Cancer Res. 2006; 66:8131–8. [PubMed: 16912191]
25. Yu WD, Ma Y, Flynn G, Muindi JR, Kong RX, Trump DL, et al. Calcitriol enhances gemcitabine anti-tumor activity in vitro and in vivo by promoting apoptosis in a human pancreatic carcinoma model system. Cell Cycle. 2010; 9:3022–9. [PubMed: 20699664]
26. Szyszka R, Grankowski N, Felczak K, Shugar D. Halogenated benzimidazoles and benzotriazoles as selective inhibitors of protein kinases CK I and CK II from Saccharomyces cerevisiae and other sources. Biochem Biophys Res Commun. 1995; 208:418–24. [PubMed: 7887958]
27. Beumer JH, Parise RA, Kanterewicz B, Petkovich M, D'Argenio DZ, Hershberger PA. A local effect of CYP24 inhibition on lung tumor xenograft exposure to 1,25-dihydroxyvitamin D(3) is revealed using a novel LC-MS/MS assay. Steroids. 2012; 77:477–83. [PubMed: 22285938]
28. Kahraman M, Sinishtaj S, Dolan PM, Kensler TW, Peleg S, Saha U, et al. Potent, selective and low-calcemic inhibitors of CYP24 hydroxylase: 24-sulfoximine analogues of the hormone 1alpha, 25-dihydroxyvitamin D(3). J Med Chem. 2004; 47:6854–63. [PubMed: 15615534]
29. Krek W, Maridor G, Nigg EA. Casein kinase II is a predominantly nuclear enzyme. J Cell Biol. 1992; 116:43–55. [PubMed: 1730748]
30. Faust M, Montenarh M. Subcellular localization of protein kinase CK2. A key to its function? Cell Tissue Res. 2000; 301:329–40. [PubMed: 10994779]

31. Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene. 2001; 20:3247–57. [PubMed: 11423974]
32. Laramas M, Pasquier D, Filhol O, Ringeisen F, Descotes JL, Cochet C. Nuclear localization of protein kinase CK2 catalytic subunit (CK2alpha) is associated with poor prognostic factors in human prostate cancer. Eur J Cancer. 2007; 43:928–34. [PubMed: 17267203]
33. Oc P, Rusch V, Talbot SG, Sarkaria I, Viale A, Socci N, et al. Casein kinase II alpha subunit and C1-inhibitor are independent predictors of outcome in patients with squamous cell carcinoma of the lung. Clinical cancer research: an official journal of the American Association for Cancer Research. 2004; 10:5792–803. [PubMed: 15355908]
34. Gapany M, Faust RA, Tawfic S, Davis A, Adams GL, Ahmed K. Association of elevated protein kinase CK2 activity with aggressive behavior of squamous cell carcinoma of the head and neck. Mol Med. 1995; 1:659–66. [PubMed: 8529132]
35. Gotz C, Bachmann C, Montenarh M. Inhibition of protein kinase CK2 leads to a modulation of androgen receptor dependent transcription in prostate cancer cells. The Prostate. 2007; 67:125–34. [PubMed: 17044081]
36. Hessenauer A, Montenarh M, Gotz C. Inhibition of CK2 activity provokes different responses in hormone-sensitive and hormone-refractory prostate cancer cells. International journal of oncology. 2003; 22:1263–70. [PubMed: 12738992]
37. Khorchide M, Lechner D, Cross HS. Epigenetic regulation of vitamin D hydroxylase expression and activity in normal and malignant human prostate cells. J Steroid Biochem Mol Biol. 2005; 93:167–72. [PubMed: 15860259]
38. Komagata S, Nakajima M, Takagi S, Mohri T, Taniya T, Yokoi T. Human CYP24 catalyzing the inactivation of calcitriol is post-transcriptionally regulated by miR-125b. Mol Pharmacol. 2009; 76:702–9. [PubMed: 19570947]
39. Lehen'kyi V, Raphael M, Oulidi A, Flourakis M, Khalimonchyk S, Kondratskyi A, et al. TRPV6 determines the effect of vitamin D3 on prostate cancer cell growth. PLoS One. 2011; 6:e16856. [PubMed: 21347289]
40. Lehen'kyi V, Flourakis M, Skryma R, Prevarskaya N. TRPV6 channel controls prostate cancer cell proliferation via Ca(2+)/NFAT-dependent pathways. Oncogene. 2007; 26:7380–5. [PubMed: 17533368]
41. Ly LH, Zhao XY, Holloway L, Feldman D. Liarozole acts synergistically with 1alpha,25-dihydroxyvitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase activity. Endocrinology. 1999; 140:2071–6. [PubMed: 10218956]
42. Zhao XY, Ly LH, Peehl DM, Feldman D. Induction of androgen receptor by 1alpha,25-dihydroxyvitamin D3 and 9-cis retinoic acid in LNCaP human prostate cancer cells. Endocrinology. 1999; 140:1205–12. [PubMed: 10067845]
43. Guzey M, DeLuca HF. A group of deltanoids (vitamin D analogs) regulate cell growth and proliferation in small cell carcinoma cell lines. Research communications in molecular pathology and pharmacology. 1997; 98:3–18. [PubMed: 9434311]
44. Bremmer F, Thelen P, Pottek T, Behnes CL, Radzun HJ, Schweyer S. Expression and function of the vitamin D receptor in malignant germ cell tumour of the testis. Anticancer research. 2012; 32:341–9. [PubMed: 22213325]
45. Jiang F, Li P, Fornace AJ Jr, Nicosia SV, Bai W. G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. J Biol Chem. 2003; 278:48030–40. [PubMed: 14506229]
46. Tront JS, Hoffman B, Liebermann DA. Gadd45a suppresses Ras-driven mammary tumorigenesis by activation of c-Jun NH2-terminal kinase and p38 stress signaling resulting in apoptosis and senescence. Cancer Res. 2006; 66:8448–54. [PubMed: 16951155]
47. Jurutka PW, Hsieh JC, MacDonald PN, Terpening CM, Haussler CA, Haussler MR, et al. Phosphorylation of serine 208 in the human vitamin D receptor. The predominant amino acid phosphorylated by casein kinase II, in vitro, and identification as a significant phosphorylation site in intact cells. J Biol Chem. 1993; 268:6791–9. [PubMed: 8384219]

48. Jurutka PW, Terpening CM, Haussler MR. The 1,25-dihydroxy-vitamin D3 receptor is phosphorylated in response to 1,25-dihydroxy-vitamin D3 and 22-oxacalcitriol in rat osteoblasts, and by casein kinase II, in vitro. Biochemistry. 1993; 32:8184–92. [PubMed: 8394128]

49. Jurutka PW, Hsieh JC, Nakajima S, Haussler CA, Whitfield GK, Haussler MR. Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. Proc Natl Acad Sci U S A. 1996; 93:3519–24. [PubMed: 8622969]

50. Arriagada G, Paredes R, Olate J, van Wijnen A, Lian JB, Stein GS, et al. Phosphorylation at serine 208 of the 1alpha,25-dihydroxy Vitamin D3 receptor modulates the interaction with transcriptional coactivators. J Steroid Biochem Mol Biol. 2007; 103:425–9. [PubMed: 17368182]

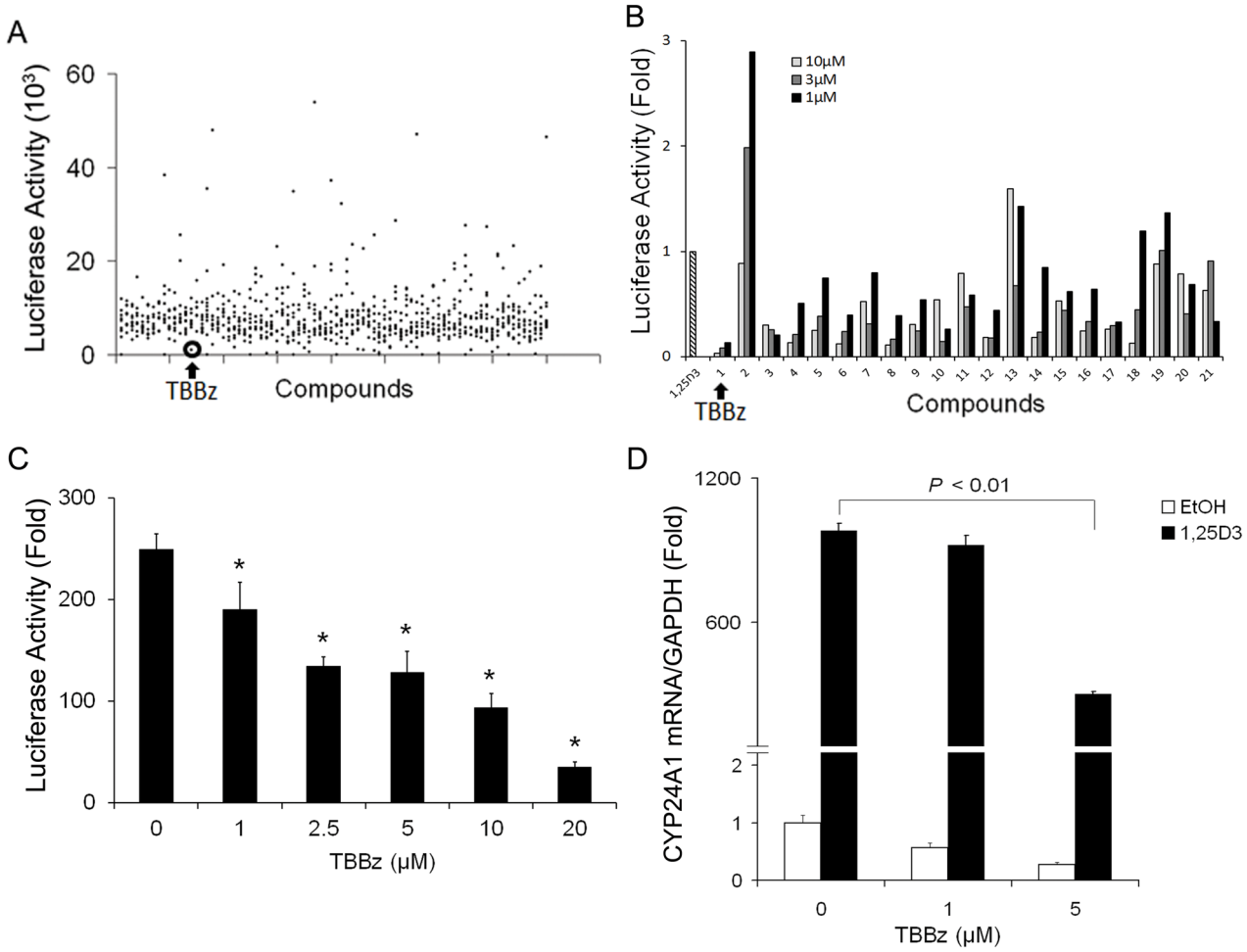

Figure 1. Identification of *CYP24A1* small molecular inhibitors by screening LOPAC compounds

(A) PC3/*CYP24A1* cells containing CYP24A1 promoter-driving luciferase were seeded into 96-well plates overnight. The LOPAC^{1280} library of pharmacologically active compounds was dispensed at a final concentration of 10 μM per compound followed by the addition of 100 nM 1,25D_3 for 24 hours. Luciferase activity for each well was assayed and luminescence measured. Each dot represents the value of luminescence. (B) Excluding the hits with high toxicity, known from SMSC database, 21 selected compounds were subjected to secondary dose-response experiments to confirm initial observations.) PC3/*CYP24A1* cells were treated with compounds at indicated concentration followed by 1,25D_3. *CYP24A1* promoter luciferase activity was measured and fold change of luciferase value was calculated for the ratio of (1,25D_3-induced luciferase activity in the presence of the compound) to (1,25D_3-induced luciferase activity in the absence of the compound). (C) PC3 cells were transfected with the *CYP24A1* promoter constructs along with *Renilla* luciferase control construct. Twenty-four hours post transfection, cells were treated with TBBz as indicated and 1,25D_3 (100 nM) for additional 24 hours and harvested, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System. The experiment was repeated twice to confirm the reproducibility of results. (*, $P < 0.05$). (D) PC3 cells were treated with TBBz as indicated followed by 1,25D_3 (100 nM). Expression of *CYP24A1* mRNA was assessed by qRT-PCR and normalized to human *GAPDH* and all samples were analyzed in triplicate.

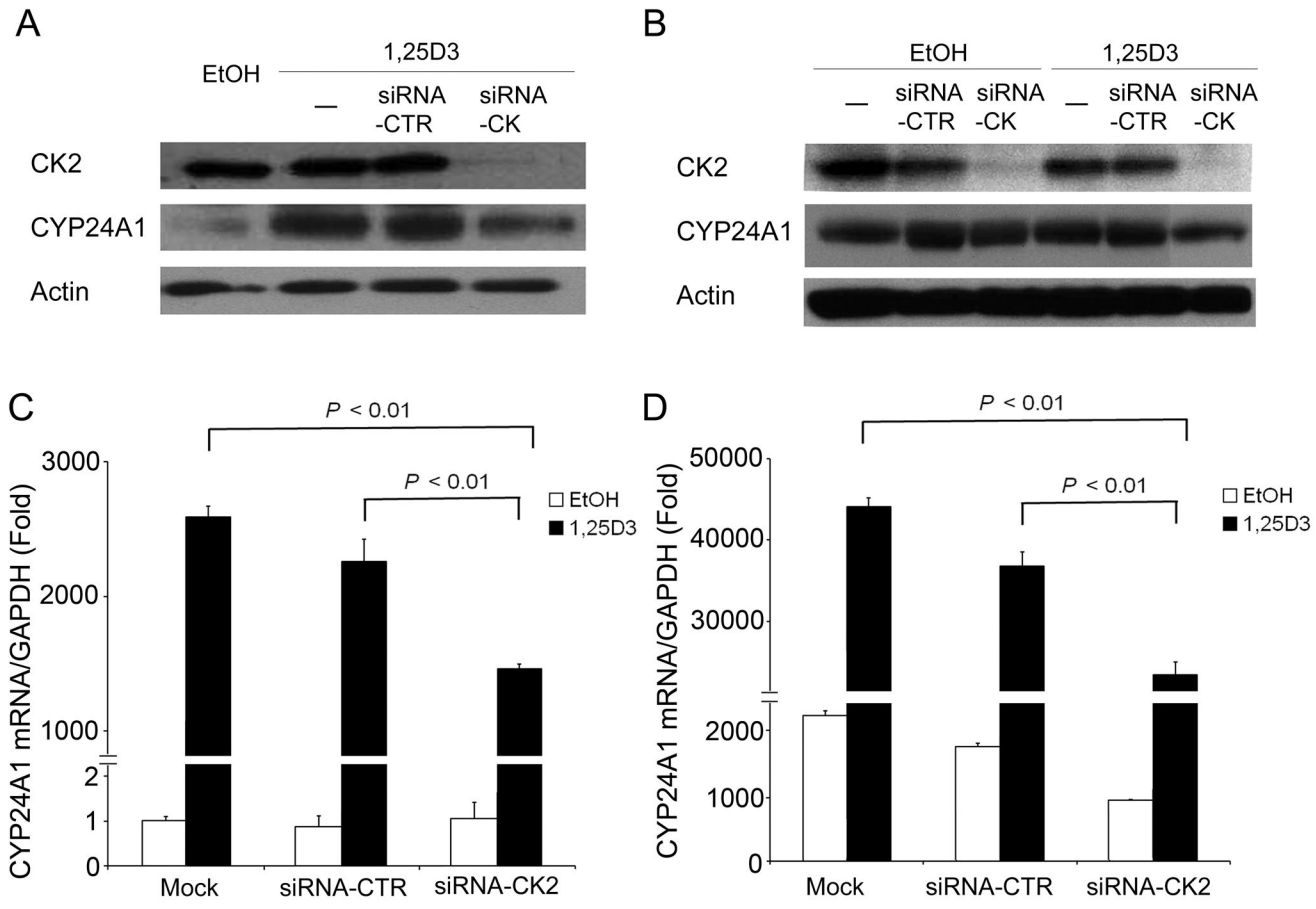

Figure 2. siRNA-mediated silencing of CK2 reduces 1,25D$_3$-induced *CYP24A1* expression

PC3 (A, C) or DU145 (B, D) cells were transfected with ON-TARGET plus SMARTpool siRNA-CK2 or siRNA control (siRNA-CTR) for 72 h. Cells were then treated with either vehicle EtOH or 1,25D$_3$ (100 nM) for 24 h or 48 h and harvested for qRT-PCR (C, D) and immunoblotting analysis (A, B).

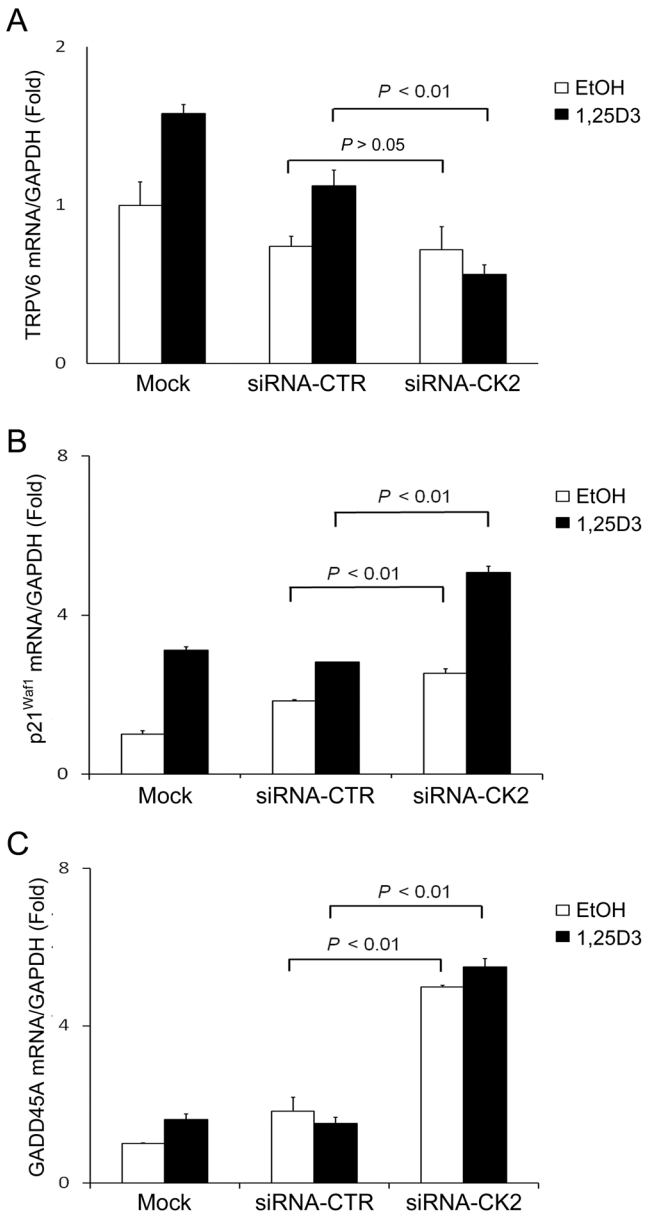

Figure 3. Effect of siRNA-CK2 on *TYPV6*, *p21*^{Waf1} and *GADD45A* mRNA expression

PC3 cells were transfected with siRNA-CK2 or siRNA-control for 72 h. Cells were then treated with either EtOH or 1,25D_3 (100 nM) for 6 hours. *TYPV6* (A), *p21*^{Waf1} (B) and *GADD45A* (C) mRNA expression were measured and normalized to human *GAPDH* and all samples were analyzed in triplicate.

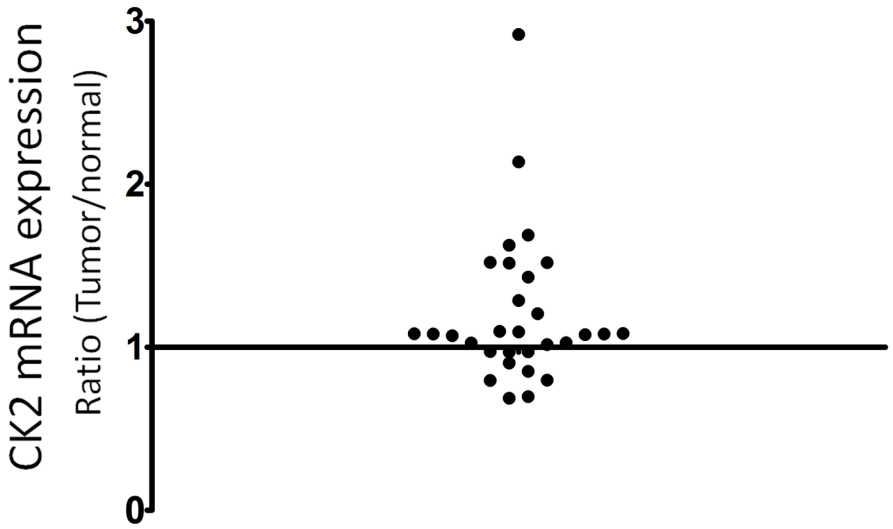

Figure 4. *CK2* expression in normal and tumor human prostate tissues

CK2 mRNA expression in human matched prostate tumor and normal lesions was measured and normalized to human *GAPDH* by qRT-PCR. The difference of CK2 mRNA expression between matched tumor and normal lesions was represented as the ratio of CK2 expression of tumor to normal lesions. Each dot represents the ratio of CK2 expression in tumor to normal lesion.

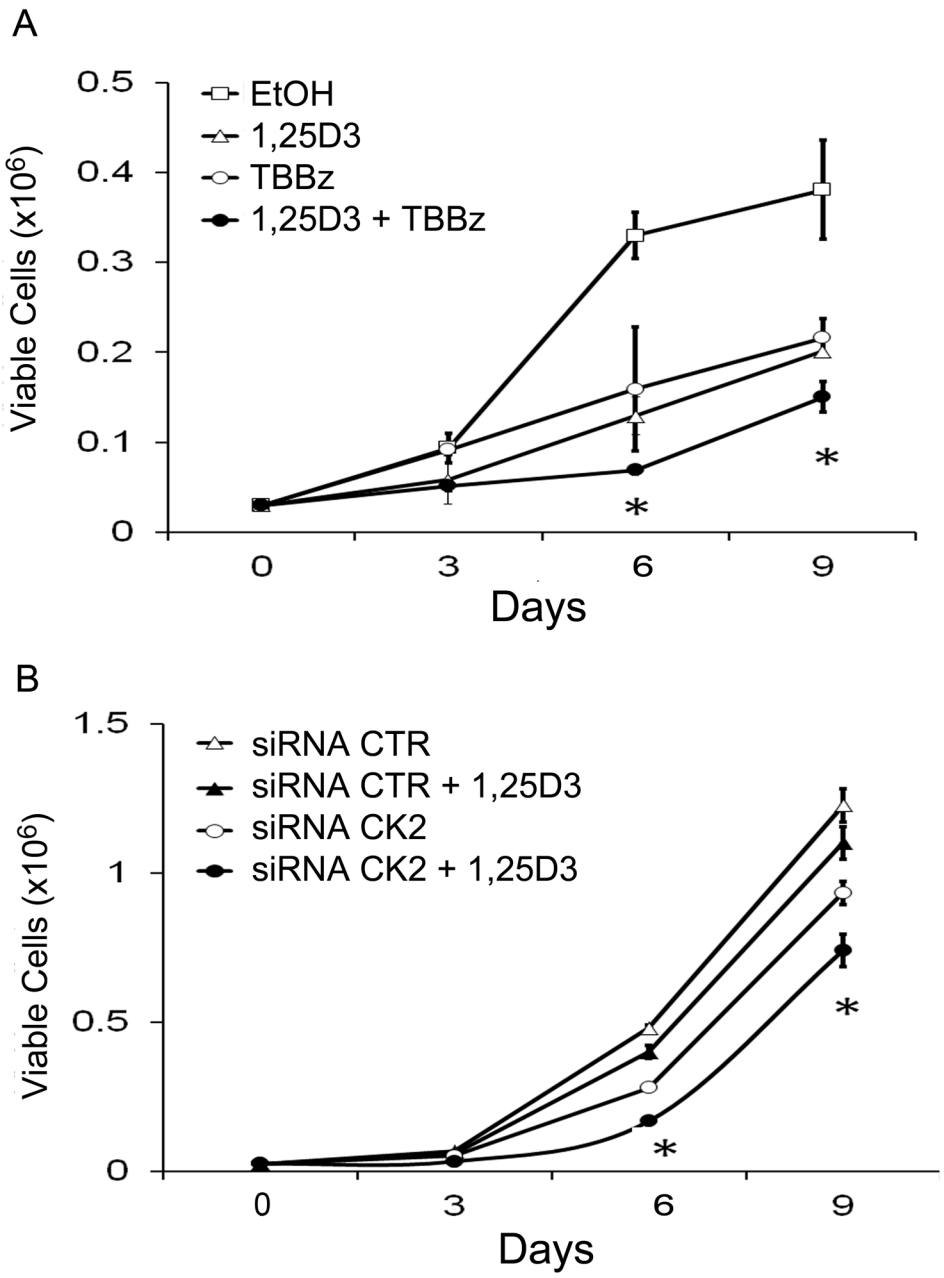

Figure 5. Enhancement of inhibitory effect of $1,25D_3$ in prostate cancer cells by TBBz or siRNA-CK2

(A) PC3 cells were treated with TBBz (5 μM), $1,25D_3$ (100 nM) or the combination of TBBz and $1,25D_3$. Viable cells were determined using trypan blue exclusion assay on day 3, 6 and 9. (B) PC3 cells were transfected with siRNA-CK2 or siRNA control for 72 h. Following transfection, cells were treated with EtOH or $1,25D_3$ (100 nM). Viable cells were determined on day 3, 6 and 9. (*, $P < 0.01$)

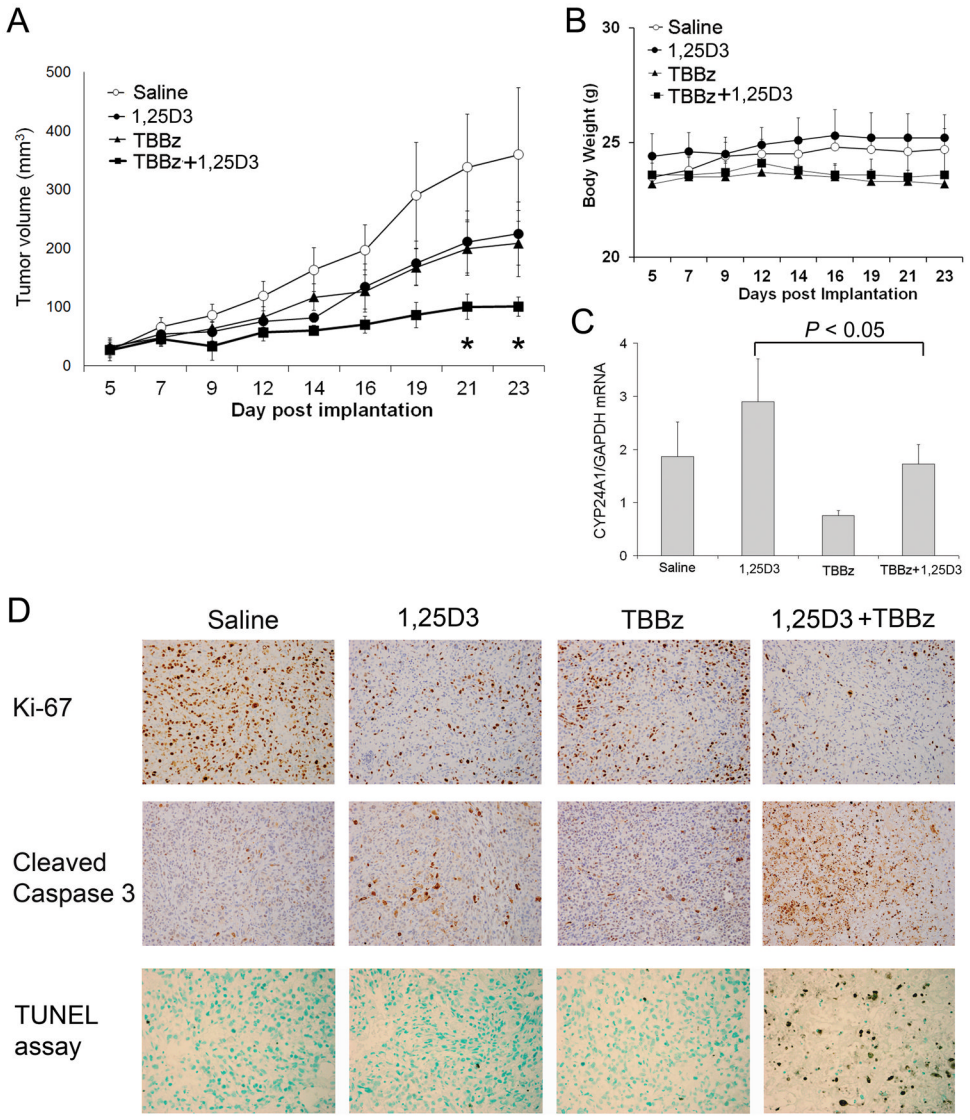

Figure 6. TBBz enhances 1,25D$_3$ anti-tumor effect in PC3 xenograft mouse model

PC3 prostate cancer cells were inoculated subcutaneously into the right flank of male SCID mice. When the tumors were palpable, animals were treated intraperitoneally with saline, 1,25D$_3$, TBBz or the combinations of 1,25D$_3$ and TBBz as described in Material and Methods. (A) Tumor growth was monitored by measuring tumor size three times per week. Tumor volumes were calculated by (length × width^2)/2. (*, $P < 0.01$). (B) Mouse weight was measured three times per week. (C) PC3 tumors were harvested after the treatment, and *CYP24A1* mRNA expression in tumor tissues was determined by qRT-PCR. (D) PC3 tumors were harvested after the treatment, and immunohistochemical staining of Ki-67 and cleaved Caspase-3 in tissues was performed. Nuclear DNA fragmentation in situ was detected using TACS-XL In Situ Apoptosis Detection Kit in tumor tissues (× 200).

Table 1

mRNA expression of CYP24A1 and CK2 in 30 human prostate tumors compared to normal lesion

Fold change	**CK2**	
	≥ 1.5	**< 1.5**
CYP24A1		
≥ 1.5	4	3
< 1.5	3	20