Inhibition of Protein Kinase CK2 Reduces Cyp24a1 Expression and Enhances 1,25-Dihydroxyvitamin D₃ Antitumor Activity in Human Prostate Cancer Cells

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Abstract

Vitamin D has broad range of physiological functions and antitumor effects. 24-Hydroxylase, encoded by the CYP24A1 gene, is the key enzyme for degrading many forms of vitamin D including the most active form, 1,25D₃. Inhibition of CYP24A1 enhances 1,25D₃ antitumor activity. 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 1,25D₃ in prostate cancer cells. In addition, TBBz downregulates endogenous CYP24A1 mRNA level in TBBz-treated PC3 cells. Furthermore, siRNA-mediated CK2 knockdown reduces 1,25D₃-induced CYP24A1 mRNA expression in PC3 cells. These results suggest that CK2 contributes to 1,25D₃-mediated target gene expression. Finally, inhibition of CK2 by TBBz or CK2 siRNA significantly enhances 1,25D₃-mediated antiproliferative effect in vitro and in vivo in a xenograft model.


Introduction

The most physiologically active form of the prohormone, vitamin D₃ (cholecalciferol), is 1,25-dihydroxyvitamin D₃ (1,25D₃). 1,25D₃ 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). Nonclassical roles for 1,25D₃ include the regulation of proliferation, differentiation, and immune function have now been identified in a variety of cell types (3). The serum level of 1,25D₃ is highly regulated through synthesis facilitated by 1α-hydroxylase (CYP27B1), and through inactivation by 24-hydroxylase (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 1,25D₃, VDR/RXR heterodimers bind to these VDREs and repress transcription through interactions with a corepressor complex that has histone deacetylase activity (7). In the presence of 1,25D₃, the corepressor complex is released, permitting the recruitment of a coactivator complex that leads to the activation of the gene (8, 9). 1,25D₃ also stimulates rapid nongenomic 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 intratumoral levels of CYP24A1 would lead to rapid degradation of 1,25D₃, thus limiting the amount of 1,25D₃ locally in the tumor cells and abrogating the antiproliferative or prodifferentiation effects of 1,25D₃ (10, 16, 17). Inhibition of CYP24A1 is expected to slow the catabolism of 1,25D₃, thereby enhancing the antiproliferative effect of 1,25D₃ (18–21). Administration of 1,25D₃ in combination with a CYP24A1 inhibitor enhances the antitumor activity of 1,25D₃ (19, 22). However, most of the current CYP24A1 inhibitors, such as ketoconazole, are relatively nonspecific, and strikingly increase the CYP24A1 expression level compared with cells treated with 1,25D₃ alone (19).

In this 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 1,25D₃-mediated function through CYP24A1 expression.
Materials and Methods

Materials

1,25D3 was purchased from Tetrionics. 25D3, LOPAC1280, and 4,5,6,7-tetrabromobenzimidazole (TBBz) were obtained from Sigma-Aldrich. The dual-luciferase assay kit was supplied by Promega. Mouse anti-CYP24 antibody was a gift from Cytochrome Inc. Anti-CK2α (H-286, sc-9030) and anti-actin antibodies were from Santa Cruz Biotechnology. Anticleaved Caspase-3 (Asp175, #9661) antibody was purchased from Cell Signaling Technology. Anti-Ki-67 antibody was purchased from Leica Microsystems (NCL-Ki67p). TaqMan Gene Expression Assay for CYP24A1 (Hs00169799_m1), CYP24A1 (Hs00751002_s1), CDKN1A (Hs00355782_m1), Growth arrest and DNA-damage-inducible protein 45α (GADD45A, Hs00169255_m1), and the transient receptor potential vanilloid type 6 gene (TRPV6, Hs00367960_m1) were purchased from Applied Biosystems. ON-TARGET plus SMARTpool siRNA specific for human CSNK2A1 (CK2α1, L-003475), ON-TARGET plus Nontargeting Pool (D-001810), and DharmaFECT 2 transfection reagents were purchased from Dharmacon. Human RNA p21Waf1 expression in paired normal and tumor human prostate 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 DNA profiling and cytogenetic analysis. Cells were maintained in culture according to providers’ protocols for a maximum of 10 passages (1 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 done by Small Molecule Screening Core (SMSC) Facility at the Roswell Park Cancer Institute using LOPAC1280 library. PC3/CYP24A1 cells were seeded to 96-well plate (10^4/well) for 24 hours and treated with 5 μmol/L of TBBz or/and 100 nmol/L of 1,25D3 or 1,000 nmol/L of 25D3. Cells were trypsinized and viable cell count was monitored 3 times per week. Body weight and tumor size was monitored 3 times per 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 anticleaved Caspase-3 was conducted as described previously (24).

Trypan blue exclusion assay

PC3 cells (2 × 10^6) were inoculated subcutaneously into the right flank of male severe combined immunodeficient (SCID) mice (6–8 weeks old). At day 8 to 9 postimplantation, when the tumors were palpable (6.5 × 5 mm), animals were treated with 1,25D3 (15.5 μg/kg/d × 3, i.p. weekly), TBBz 3 times weekly (15 mg/kg/d, i.p., every 2 days), or the combination for 2 weeks.

Tumor growth assay

PC3 cells (2 × 10^6) were inoculated subcutaneously into the right flank of male severe combined immunodeficient (SCID) mice (6–8 weeks old). At day 8 to 9 postimplantation, when the tumors were palpable (6.5 × 5 mm), animals were treated with 1,25D3 (15.5 μg/kg/d × 3, i.p. weekly), TBBz 3 times weekly (15 mg/kg/d, i.p., every 2 days), or the combination for 2 weeks. Body weight and tumor size was monitored 3 times per week.

Statistics

Statistical significance of data was determined by 2-tailed Student t test. Wilcoxon signed rank test was done to compare the expression levels of CK2 in paired normal and tumor samples. 2 × 2 contingency table was conducted to analyze the correlation between increased CYP24A1 expression and CK2 expression in paired normal and tumor human prostate samples.

Quantitative reverse transcriptase PCR (qRT-PCR)

Expression of CK2, CYP24A1, TRPV6, p21Waf1, 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.

Materials and Methods
Results

Screening of small molecules from LOPAC1280 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 LOPAC1280 library in this system resulted in the identification of 70 hits, each of which had over 50% inhibition of 1,25D3-induced CYP24A1 promoter activity (Fig. 1A). Excluding the hits with high toxicity, known from SMSC database, 21 selected molecules were subjected to secondary dose–response experiments to confirm initial observations. Seventeen hits reduced 1,25D3-mediated CYP24A1 promoter activation (Fig. 1B). Among them, 4,5,6,7-TBBz displayed the strongest inhibitory effect and was chosen for further investigation (Fig. 1A and B).

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 1,25D3-regulated CYP24A1 expression. PC3 cells were treated with 1 or 5 μmol/L of TBBz alone or followed by 10, 30, and 100 nmol/L of 1,25D3. qRT-PCR results showed that PC3 cells displayed low endogenous CYP24A1 mRNA level and dose-dependent induction of CYP24A1 mRNA expression by 1,25D3 (Supplementary Fig. S1A). TBBz significantly (P < 0.01) reduced 1,25D3-induced CYP24A1 mRNA expression in a dose-dependent manner (Fig. 1D). Less induction of CYP24A1 expression by lower dose of 1,25D3 was relatively less influenced by TBBz (Supplementary Fig. S1A). These results indicate that TBBz inhibits endogenous and 1,25D3-induced CYP24A1 expression at the transcriptional level. Furthermore, we observed that 25D3, the precursor to 1,25D3 also induced CYP24A1 mRNA expression in PC3 cells and TBBz inhibits 25D3-induced CYP24A1 expression at the transcriptional level in PC3 cells (Supplementary Fig. S2A).
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 1,25D₃. 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 with control siRNA (Fig. 2A and B). 1,25D₃ significantly \((P < 0.01)\) increased CYP24A1 expression in PC3 cells, which express a low level of endogenous CYP24A1 (Fig. 2A and C, respectively), and in DU145 cells, which display a high level of endogenous CYP24A1 (Fig. 2B and D, respectively). Transfection with siRNA-CK2 significantly reduced 1,25D₃-induced CYP24A1 expression at both mRNA and protein level in PC3 (Fig. 2A and C) and DU145 cells (Fig. 2B and D) as compared with the mock-transfected or siRNA control transfected samples. These results indicate that CK2 plays a role in regulation of 1,25D₃-induced CYP24A1 expression.

Differential effects of siRNA-CK2 on 1,25D₃-induced TRPV6, p21Waf1, and GADD45A mRNA expression

To investigate whether silencing of CK2 affects the expression of other vitamin D target genes, TRPV6, p21Waf1, and GADD45A mRNA expression was measured by qRT-PCR in CK2 knockdown PC3 cells. 1,25D₃ induced TRPV6, p21Waf1, and GADD45A mRNA expression. siRNA-CK2 significantly \((P < 0.01)\) reduced 1,25D₃-induced TRPV6 mRNA expression as compared with the mock-transfected or siRNA control transected cells (Fig. 3A). In contrast, knockdown of CK2 markedly increased p21Waf1 and slightly increased GADD45A mRNA expression (Fig. 3B and C). 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 with normal lesions \((P = 0.0224; \text{Fig. 4})\). 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 with normal samples, we built a \(2 \times 2\) contingency table by dividing the 30 samples based on the CYP24A1 expression change (\(\geq 1.5\) fold up vs. other) and CK2 expression change (\(\geq 1.5\) fold up vs. other). Seven samples display increased CYP24A1 expression in a total of 30 prostate tumors compared with 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 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 antiproliferative activity by TBBz or siRNA-CK2

To ascertain the potential therapeutic role of CK2 inhibitors in 1,25D₃ antitumor action, cell viability and cell proliferation...
was examined using the Trypan blue exclusion assay after treatment with 1,25D₃, TBBz, or the combination of 1,25D₃ and TBBz for 9 days. Combination treatment of 1,25D₃ and TBBz resulted in a significant (P < 0.05) enhancement of 1,25D₃ antiproliferative effect in PC3 cells (Fig. 5B). This indicates that CK2 inhibition is antiproliferative and enhances 1,25D₃ antiproliferative effect.

Enhancement of 1,25D₃ antitumor activity by TBBz in vivo

Having showed the efficacy of the combination treatment of 1,25D₃ 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 1,25D₃ and TBBz, compared with 1,25D₃ or TBBz alone (Fig. 6A). Mice grew normally without suffering from weight loss at a dose sufficient to induce antitumor effect (Fig. 6B). These results indicate that CK2 inhibitor TBBz enhances 1,25D₃ antitumor activity in vivo.

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

To investigate the effect of TBBz on CYP24A1 expression in vivo, tumor tissues were harvested at the end of the treatment as described in Fig. 5A, and CYP24A1 mRNA expression was measured by qRT-PCR. 1,25D₃ increased CYP24A1 expression and TBBz reduced CYP24A1 expression in tumors as compared with saline group (Fig. 6C). Furthermore, TBBz significantly (P < 0.05) reduced 1,25D₃-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 antitumor activity of 1,25D₃ 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, 1,25D₃ or TBBz reduced Ki-67 staining (Fig. 6D). The combination of 1,25D₃ and TBBz further reduced Ki-67 staining (Fig. 6D). We also observed that saline group did not have positive cleaved caspase-3 staining, whereas 1,25D₃ or TBBz treatment alone induced caspase-3 cleavage in the tumor tissue (Fig. 6D). The combination of 1,25D₃ and TBBz further enhanced caspase-3 cleavage (Fig. 6D). We also observed that saline group did not have positive cleaved caspase-3 staining, whereas 1,25D₃ or TBBz treatment alone induced caspase-3 cleavage in the tumor tissue (Fig. 6D). The combination of 1,25D₃ and TBBz further enhanced caspase-3 cleavage (Fig. 6D). The effect of 1,25D₃ and TBBz on apoptosis was further confirmed by TUNEL assay (Fig. 6D). These results clearly indicate the potential usefulness of the combination of 1,25D₃ 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 antitumor 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 1,280 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 1,25D₃ antitumor 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 tumors compared with 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 1,25D₃.

CYP24A1 expression is heterogeneous in prostate cancer (23). It is noteworthy that 3 human prostate tumor samples with high CK2 expression did not express high level of CYP24A1 and 3 prostate tumor samples with high CYP24A1 expression did not express high level of CK2 compared with 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

| Table 1. mRNA expression of CYP24A1 and CK2 in 30 human prostate tumors compared with normal lesion |
|-------------------------------------------------|--------|------|
| CK2 | CYP24A1 |  |  |  |
| Fold change |  |  |  |
| ≥1.5 | 4 | 3 |
| <1.5 | 3 | 20 |
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. Upregulation of TRPV6 by 1,25D_{3} in prostate cancer cells is considered to be proproliferative by increasing Ca^{2+} uptake (39, 40). However, siRNA-CK2 enhanced 1,25D_{3}-mediated induction of p21^{Waf1} and GADD45A. p21^{Waf1} is accounted in part for the antiproliferative 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 G_{2}/M cell-cycle arrest (45, 46). In this 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 cooperative 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 cotransfected 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 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

Figure 6. TBBz enhances 1,25D_{3} antitumor 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 Materials and Methods. A, tumor growth was monitored by measuring tumor size 3 times per week. Tumor volumes were calculated by (length \times width^{2})/2 (*, P < 0.01). B, mouse weight was measured 3 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 conducted. Nuclear DNA fragmentation in situ was detected using TACS-XL In Situ Apoptosis Detection Kit in tumor tissues (> 200).
D target genes. CK2 inhibitor TBBz significantly enhances 1,25\(\text{D}_3\) antitumor activity in vitro and in vivo. These findings provide support for the combination treatment of CK2 inhibitor and vitamin D in prostate cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: W. Luo, W.-D. Yu, Y. Ma, M. Chernov, C. S. Johnson
Development of methodology: W. Luo, W.-D. Yu, M. Chernov
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.-D. Yu, M. Chernov
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Luo, W.-D. Yu, M. Chernov
Writing, review, and/or revision of the manuscript: W. Luo, W.-D. Yu, Y. Ma, D. L. Trump

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