CYP24A1 Inhibition Enhances the Antitumor Activity of Calcitriol

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High systemic exposures to calcitriol are necessary for optimal antitumor effects. Human prostate cancer PC3 cells are insensitive to calcitriol treatment. Therefore, we investigated whether the inhibition of 24-hydroxylase (CYP24A1), the major calcitriol inactivating enzyme, by ketoconazole (KTZ) or RC2204 modulates calcitriol serum pharmacokinetics and biologic effects. Dexamethasone (Dex) was added to minimize calcitriol-induced hypercalcemia and as a steroid replacement for the KTZ inhibition of steroid biosynthesis cytochrome P450 enzymes. KTZ effectively inhibited time-dependent calcitriol-inducible CYP24A1 protein expression and enzyme activity in PC3 cells and C3H/HeJ mouse kidney tissues. Systemic calcitriol exposure area under the curve was higher in mice treated with a combination of calcitriol and KTZ than with calcitriol alone. KTZ and Dex synergistically potentiated calcitriol-mediated antiproliferative effects in PC3 cells *in vitro*; this effect was associated with enhanced apoptosis. After treatment with calcitriol and KTZ/Dex, although caspase-9 and caspase-3 were not activated and cytochrome *c* was not released by mitochondria, caspase-8 was activated and the truncated Bid protein level was increased. Translocation of apoptosis-inducing factor to the nucleus was observed, indicating a role of the apoptosis-inducing factor-mediated and caspase-independent apoptotic pathways. Calcitriol and KTZ/Dex combination suppressed the clonogenic survival and enhanced the growth inhibition observed with calcitriol alone in PC3 human prostate cancer xenograft mouse model. Our results show that the administration of calcitriol in combination with CYP24A1 inhibitor enhances antiproliferative effects, increases systemic calcitriol exposure, and promotes the activation of caspase-independent apoptosis pathway. **(*Endocrinology* 151: 4301–4312, 2010)**

Calcitriol (1,25-dihydroxycholecalciferol), the biologically most active form of vitamin D, has significant antiproliferative activities in a variety of human cancer cells *in vitro* and *in vivo* in murine, rat, canine, and human xenograft tumor models (1). The molecular mechanisms for these calcitriol effects are not fully understood but include G_0-G_1 cell cycle arrest, induction of apoptosis, and cell differentiation (1). Recent studies suggest that calcitriol may also exert antitumor activity by targeting tumor endothelial cells and angiogenesis (2–4). In preclinical models, high doses of calcitriol are required to elicit antitumor activity (5, 6) and indicate that an intermittent schedule of administration of high calcitriol doses is safe and has striking antitumor effects in murine squamous cell carcinoma (SCCVII/SF) and canine sarcoma model systems (7, 8).

Clinical trials in cancer patients have shown that intermittent administration of high-dose calcitriol is safe and the therapeutic activity of calcitriol alone, calcitriol + dexamethasone (Dex) and calcitriol + docetaxel has been suggested in castration-resistant and castration-sensitive prostate cancer patients as assessed by change in serum prostate-specific antigen levels as a biomarker of response and survival (9–12). Commercially available calcitriol

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Abbreviations: AIF, Apoptosis-inducing factor; AUC, area under the concentration-time curve; Bid, BH3 interacting domain death agonist; calcitriol, 1,25-dihydroxycholecalciferol; CI, combination index; CL/F, apparent clearance; COX IV, cytochrome *c* oxidase subunit IV; CYP24A1, 24-hydroxylase; Dex, dexamethasone; EtOH, ethyl alcohol; KTZ, ketoconazole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 24-OHase, 24-hydroxylase; PK, pharmacokinetics; p.o., *per os*; qd, once daily; tBid, truncated Bid.

formulations are associated with poor bioavailability and substantial interpatient variability in serum calcitriol peak levels and systemic exposure (13–15). These calcitriol pharmacokinetics (PK) characteristics are a barrier to achieving the high systemic exposures that are necessary for optimal antitumor effects. Poor bioavailability and interpatient variability in levels could be explained at least in part by local and systemic catabolism of vitamin D_3.

24-Hydroxylase (CYP24A1), also called 24-OHase, a mitochondrial cytochrome P450, is the primary vitamin D_3-inactivating enzyme (16, 17). CYP24A1 is constitutively expressed in kidney and gastrointestinal mucosa and in most tissues is transcriptionally induced by calcitriol (16, 17). By stimulating calcitriol degradation, CYP24A1 limits calcitriol biologic activity. Increased expression of CYP24A1 in several human cancer tissues (18–20) suggests that CYP24A1 could specifically contribute to the diminished efficacy of calcitriol. The inhibition of CYP24A1 has the potential not only to increase circulating serum calcitriol levels but also to inhibit intratumor degradation of calcitriol. *In vitro* studies show that inhibitors of CYP24A1 potentiate the antiproliferative effect of calcitriol in human cancer cells exhibiting CYP24A1 activity (21–23). The pharmacological and molecular mechanisms for this enhanced *in vitro* antiproliferative effects and the *in vivo* modulation of calcitriol PK and antitumor activity have not been fully investigated.

Using a nonspecific, broad inhibitor of cytochrome P450 family of enzymes, ketoconazole (KTZ), we investigated the effects of CYP24A1 inhibition on systemic exposure to calcitriol in normal C3H/HeJ mice and the antitumor activity of calcitriol in nude mice bearing human PC3 xenografts. We also examined the association of apoptosis with enhanced calcitriol antiproliferative effects by CYP24A1 inhibitor in PC3 cells *in vitro*. Our studies have focused on KTZ, an antifungal agent, because it is also widely used to treat patients with progressing prostate cancer despite androgen deprivation (24, 25). Calcitriol was administered in combination with Dex for three reasons. First, Dex is used as steroid replacement, because KTZ inhibits steroidogenic cytochrome P450 enzymes (26). Second, glucocorticoids, especially Dex, have been extensively used in the treatment of hypercalcemic states, including calcitriol-induced hypercalcemia (27, 28). Third, the administration of Dex has been shown to enhance calcitriol antitumor effects in some tumor models (6). *In vitro* and *in vivo* inhibition of CYP24A1 and modulation of calcitriol PK were also performed using RC2204, a specific CYP24A1 inhibitor, which requires no Dex administration.

Materials and Methods

Chemicals and reagents

Calcitriol (Hoffmann-La Roche, Inc., Nutley, NJ) was reconstituted in 100% ethyl alcohol (EtOH) and stored protected from light under a layer of nitrogen gas at −70 C. KTZ was purchased from Sigma-Aldrich Co. (St. Louis, MO). RC2204 was kindly provided by Sapphire Therapeutics (Bridgewater, NJ). Dex was from Elkin-Sinn, Inc. (Cherry Hill, NJ). Hydroxyvitamin 25-[26, 27-^{3}H] (-[^{3}H]-25-D_3) was from PerkinElmer Science Corp. (Boston, MA). Authentic calcitriol CYP24A1 oxidation metabolites for the HPLC preparation were kindly provided by S. Reddy and M. Uskokovic. Annexin V-phycoerythrin and 7AAD were from BD Biosciences PharMingen (San Diego, CA). Anti-CYP24A1 antibody was from Cytochroma, Inc. (Markham, Ontario, Canada). Anticaspase-3, anticaspase-9, anti-BH3 interacting domain death agonist (Bid), anticytochrome *c*, and anti-apoptosis-inducing factor (AIF) were from Cell Signaling Technology (Beverly, MA). Anti-cytochrome *c* oxidase subunit IV (COX IV) was from Molecular Probes (Eugene, OR). Antihistone H1 was from Stressgen (Victoria, British Columbia, Canada). Antiactin was from Calbiochem (San Diego, CA).

Cell culture

PC3, a human prostate cancer cell line, from the American Type Culture Collection (Manassas, VA), was cultured in F-12 Kaighn's media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin and streptomycin. EtOH was the solvent control for *in vitro* calcitriol treatment and never exceeded a final concentration of 0.0004%.

Growth inhibition assay and dose-effect analysis

Cell growth inhibition was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. PC3 cells were seeded at 7500 cells/well into 96-well tissue culture plates. Cells were treated with EtOH or various combination of calcitriol (0–6 μM) and KTZ/Dex (KTZ, 0–21 μM) for 48 h. The ratio of KTZ to Dex was fixed at 3.5:1. Cell growth was assessed by staining with 0.5% MTT, the resulting formation of crystals was dissolved in 10% sodium dodecyl sulfate and 10 mM HCL solution overnight at 37 C, and the absorbance was read with an ELISA reader at 590 nm. Drug interactions were quantitated by median-dose effect analysis, and combination index (CI) values were derived using CalcuSyn software (Biosoft, Ferguson, MO), as described previously (29). CI values of less than 1, 1, and more than 1 indicate synergism, additive, and antagonism between the drugs, respectively.

In vitro 24-OHase activity assay

PC3 cells express negligible constitutive 24-OHase activity (30). Therefore, PC3 cells were pretreated with 100 nM calcitriol for 24 h to induce 24-OHase activity. Cells were washed twice and harvested by scraping using 24-OHase buffer [0.19 M sucrose, 25 mM sodium succinate, 2 mM $MgCl_2$, 1 mM EDTA and 20 mM HEPES, CYP24A1 (pH 7.4)]. CYP24A1 enzyme activity assay consisted of 0.25 mg of PC3 cellular protein, as the CYP24A1 enzyme source, and 0.5 μCi (110,000 dpm) [^{3}H]-25-D_3 as the substrate in 0.5 ml of 24-OHase activity buffer. For the inhibition of 24-OHase activity by the enzyme, the preparation was preincubated at room temperature with either KTZ (0–30 μM) or RC2204 (0–3 μM) for 10 min, and the reaction was

initiated by the addition of substrate and incubated for additional 30 min at 37 C. [^{3}H]-25-D_3 oxidative metabolites were extracted by liquid-liquid extraction and separated by HPLC with fraction collection. Metabolites were quantitated by liquid scintillation counting of HPLC fractions as previously described (31, 32). Plots of 24-OHase activity *vs.* inhibitor concentrations were used to determine RC2204 and KTZ IC_{50} inhibition values.

Apoptosis assays

PC3 cells were treated with EtOH, 375 nM of calcitriol, 2.48 μM of KTZ, 375 nM Dex, KTZ + Dex or calcitriol + KTZ + Dex combination for 24 h. Cells were collected and double stained with phycoerythrin-conjugated annexin V and 7AAD according to manufacturer's instructions as described previously (33). Annexin V positive cells were considered apoptotic; 10,000 events were collected for each sample using a FACScan (Becton Dickinson and Company, San Jose, CA), and data were analyzed using the Winlist program (Verity House, Topsham, ME).

Caspase activity assays

The activities of caspase-3, caspase-8, and caspase-9 were measured using the caspase-family Colorimetric Assay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's protocol. Caspase activity was assessed by absorbance divided by protein loading.

Western blot analysis

Cell and tissue lysates were prepared using lysis buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8.0), 150 mM NaCl, 0.6 mM PMSF, and 5 μg/ml leupeptin], and Western blot analysis was performed as described previously (34). Mitochondrial and cytosolic fractions were isolated using Mitochondrial Fractionation kit following the manufacturer's protocol (Active Motif, Carlsbad, CA). COX IV was used as a control for mitochondrial fraction. Nuclear and cytosolic fractions were isolated using Nuclear Extract kit (Active Motif). Histone H1 was used as a control for nuclear fraction.

In vivo PK study design and analytical assay

PK studies and CYP24A1 enzyme activity studies were performed in nontumor-bearing C3H/HeJ mice. C3H/HeJ mice have been used extensively by our group to evaluate calcitriol antitumor activity in a murine model of squamous cell carcinoma (29, 34). C3H/JeH mice were treated with either calcitriol alone or a combination of calcitriol and KTZ or RC2204. Calcitriol and KTZ were ip administered at 6.25 μg/kg and 50 mg/kg doses, respectively. KTZ was administered 0.5 h before and at the time of calcitriol administration. Calcitriol and RC2204 were orally [*per os* (p.o.)] administered at 2.1 μg/kg and 200 mg/kg, respectively. RC2204 was administered 0.5 h before calcitriol administration, at the time of calcitriol and every 3.5 h thereafter for a total of five doses. The doses, route, and frequency of KTZ and RC2204 administration were based on PK and solubility profiles. RC2204 has plasma half life of approximately 2 h, is relatively insoluble in most solvents, and was administered by oral gavages as a suspension. KTZ, plasma elimination half life of approximately 8 h, was administered ip PK. Blood samples were obtained 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h after calcitriol.

Baseline calcitriol PK serum samples were obtained from four untreated mice. Three mice were treated and PK sampled at each time point. Serum sample from each individual mouse was analyzed separately. Serum samples prepared by a 10-min centrifugation at 2000 $\times$ *g* at 4 C were stored at −70 C until assayed. Serum calcitriol levels were measured using DiaSorin 1,25-dihydroxyvitamin D_3-[I^{125}] RIA kits as previously described (35). Pharmacokinetic parameters were analyzed using a noncompartmental analysis of the data (WinNonlin version 5.2; Pharsight Corp., Mountain View, CA). All PK parameters were summarized using mean and SEM. The serum PK parameters estimated were peak levels, area under the concentration-time curve (AUC) from time 0 to 24 h, and apparent clearance (CL/F = dose/AUC). Comparisons between the PK parameters for each treatment were made using the unpaired Student's *t* test. Statistical significance for all testing was set at $P < 0.05$.

In vivo pharmacodynamic effects of CYP24A1 inhibition

Pharmacodynamic studies consisted of measuring CYP24A1 protein expression and enzyme activity in kidney collected from nontumor-bearing C3H/JeH mice during the PK studies. Kidneys were collected from untreated mice and 4, 6, 24, and 48 h for the calcitriol alone, calcitriol + KTZ, or calcitriol + RC2204 treatment groups. Pieces of kidney tissues were washed with normal saline at 4 C (to remove blood) and stored at −70 C until analyzed. Tissues lysates 24-OHase activity assay, approximately 0.5 cm^3 of each mouse tissue was homogenized using a Teflon pestle homogenizer in 24-OHase buffer [0.19 M sucrose, 25 mM sodium succinate, 2 mM $MgCl_2$, 1 mM EDTA, and 20 mM HEPES buffer (pH 7.4)] at 4 C. 24-OHase activity was assayed with 0.5 mg protein of the homogenate.

In vivo effect of KTZ on calcitriol-mediated antitumor activity

In vivo effects of KTZ on calcitriol mediated antitumor activity was evaluated in a human PC3 xenograft tumor model in nude mice using a study protocol approved by the Institutional Animal Care and Use Committee. Adult homozygous nude mice (Charles River Laboratories, Charles River, MA) were sc injected with 2 $\times 10^6$ PC3 cells, log-growth phase in 0.1 ml of Matrigel + Hank's Buffered Salt Solution (1:1), in the right rear flank. At d 8–9 after implantation, when the tumors were palpable (~6.5 $\times$ 5 mm), the animals (four to five animals/group) were either untreated or ip treated with calcitriol alone [31.25 μg/kg · d, once daily (qd) $\times$ 2, weekly], KTZ alone (50 mg/kg · d, qd $\times$ 3, weekly), Dex alone (0.45 mg/kg · d, qd $\times$ 3, weekly), two drug combination (calcitriol + Dex), or three drug combination (calcitriol + KTZ/Dex). Dex was given as steroid replacement because KTZ inhibits steroidogenesis and is also known to enhance calcitriol antitumor activity (6). The animals were treated for three consecutive weeks. Two approaches were used to assess the effect of each of these treatments on PC3 tumor growth.

In vivo excision clonogenic assay

Twenty-four hours after the last treatment, the animals were killed, and their tumors were excised. Aliquots of minced tumor were enzymatically dissociated for 60 min at room temperature with a mixture of type I collagenase, deoxyribonuclease, and EDTA. For each treatment group, a series of numbers of tumor cells was plated in six-well tissue culture plates. After incubation for 11 d, colonies were counted, and the surviving fraction was calculated using the equation: surviving fraction = the cloning

efficiency of treated cells/cloning efficiency of untreated control cells. The surviving fraction per gram of tumor is defined as the number of clonogenic tumor cells per gram of treated tumor divided by the number of clonogenic tumor cells per gram of control tumor (29).

Tumor size measurements

Tumor measurements for each treatment group were obtained using calipers before initiating treatment (initial tumor volume) and two times per week for three consecutive weeks. Tumor volumes were calculated by the following formula: volume = (length × width^2)/2. For each tumor, fractional tumor volumes were calculated using the following formula: fractional tumor volume = volume on day measured/initial tumor volume (29).

Immunohistochemistry

Tissue were fixed in 10% formalin for 24 h, transferred to 70% ethanol, and then embedded in paraffin; 5-μm sections were cut and mounted on slides. Slides were hydrated through xylene and graded alcohol and equilibrated in H_2O. Antigen retrieval was performed with sodium citrate 10 mM (pH 6), using microwave for 20 min. Endogenous peroxidases were quenched with 3% H_2O_2 in methanol. Rabbit polyclonal anti-Ki-67 (NCL-Ki67p; Novocastra, Bannockburn, IL) or mouse monoclonal anticleaved caspase-3 (no. 9661; Cell Signaling Technology) was used for immunohistochemical staining of tumor tissue sections at dilutions 1:1000 and 1:100, respectively. All the slides were then washed several times in Tris-PO_4 and incubated with horseradish peroxidase swine antirabbit (1:100) or rabbit antimouse IgG (1:200) (Dako, Carpinteria, CA). The antigen was visualized by a 15-min incubation with 9 diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO). Negative controls, made by excluding antibodies from the reaction, showed no specific staining. Counterstaining was performed with hematoxylin (Richard-Allan Scientific, Waltham, MA), and cover slips were mounted with Permount (Fisher, Pittsburgh, PA).

Other analytical assays

Serum calcium and creatinine levels were measured using QuantiChrom Calcium Assay kit (DICA-500) (Bioassay Systems, Hayward, CA) and Creatinine Assay kits (BioVision Research Products, Mountain View, CA), respectively. Quantitative serum total testosterone levels were determined using Testosterone Enzyme Immunoassay Test kits from Prolias Technologies (Rockville, MD).

Data analysis and statistical methods

The Wilcoxon rank sum test and/or ANOVA analysis were used to compare baseline, time-dependent, and calcitriol-induced changes in 24-OHase activity as appropriate. Statistical significance of data was determined by ANOVA, Wilcoxon signed rank test, or unpaired Student's *t* test as appropriate. A $P < 0.05$ was considered significant. All statistical calculations were performed using NCSS statistical software (Kaysville, UT).

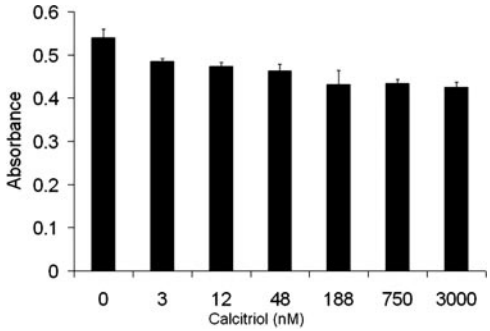

FIG. 1. PC3 cells are insensitive to calcitriol-mediated antiproliferative effects. Cell growth inhibition was measured by MTT assay after 48 h of treatment with 0–3000 nM calcitriol.

Results

PC3 cells are insensitive to calcitriol treatment

Calcitriol has been reported to have growth inhibitory effects in a number of cancer cells. To examine whether calcitriol inhibits the growth of prostate cancer PC3 cells, they were treated with various concentrations of calcitriol. The results of MTT assays showed that calcitriol, even at high doses, did not markedly inhibit PC3 cell growth (Fig. 1), indicating that these PC3 cells are insensitive to calcitriol treatment.

KTZ and RC2204 inhibit calcitriol-induced 24-OHase activity in PC3 cells

CYP24A1 is a specific metabolizing enzyme of calcitriol. To examine whether the resistance of PC3 cells to calcitriol was associated with the inactivating activity of CYP24A1, we examined the effect of CYP24A1 inhibition on calcitriol metabolism. PC3 cells exhibit no constitutive 24-OHase activity. We examined the effect of calcitriol on CYP24A1 induction (Fig. 2, A and B). Maximum calcitriol-induced 24-OHase activity in PC3 cells was observed in cells treated with 100 nM calcitriol for 24 h. We tested the inhibition of calcitriol-induced 24-OHase activity in PC3 cells on 25-hydroxyvitamin D_3 oxidative metabolism (25-D_3) using KTZ and RC2204. The KTZ and RC2204 dose-response curves for the inhibition of inducible 24-OHase activity are shown in Fig. 2C. In PC3 cells, RC2204 is a more potent inhibitor of CYP24A1 enzyme activity than KTZ. The IC_{50} inhibition values for RC2204 and KTZ were 47.9 nM and 1.45 μM, respectively.

In vivo inhibition of 24-OHase activity in normal C3H/HeJ mice tissues

The *in vivo* inhibition of CYP24A1 activity was evaluated by measuring 24-OHase activity in kidney tissues obtained from untreated, calcitriol-, calcitriol + KTZ/Dex-, and Calcitriol + RC2204-treated normal C3H/HeJ mice. Time-dependent increase in CYP24A1 protein expression (Fig. 2D) and 24-OHase activity (Fig. 2E) was

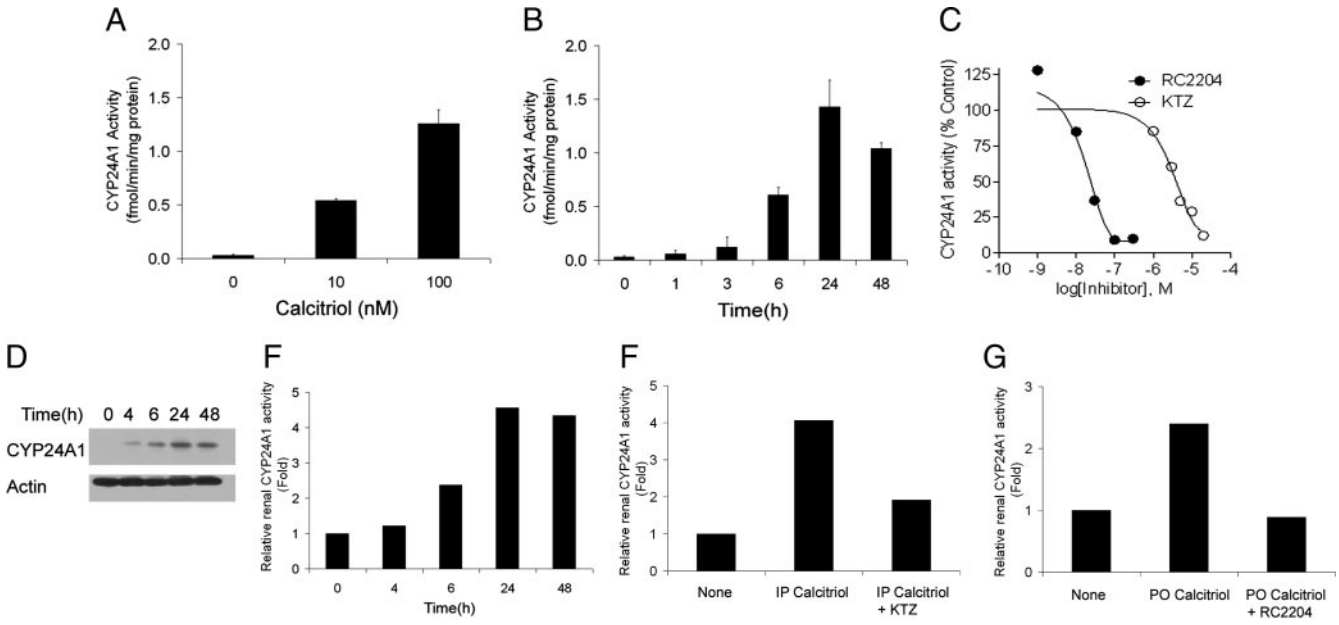

FIG. 2. *In vitro* and *in vivo* induction and inhibition of CYP24A1 enzyme activity. A, Dose response for the calcitriol-induced CYP24A1 activity in PC3 cells. B, Time course for 100 nM calcitriol induced CYP24A1 activity in PC3 cells. C, KTZ and RC2204 dose-response curves for the inhibition of calcitriol induced CYP24A1 activity in PC3 cells treated with 100 nM calcitriol for 24 h. D, Time-dependent changes in CYP24A1 protein expression were measured by Western blot analysis in kidney tissues collected from normal C3H/JeH mice treated with a single ip dose of 6.25 μg/kg calcitriol. E, Time-dependent changes in mice kidney tissue CYP24A1 activity after a single ip dose of 6.25 μg/kg calcitriol. F, CYP24A1 activity measured in C3H/JeH mice kidney collected 24 h after treatment with single ip dose of 6.25 μg/kg calcitriol alone and from mice pretreated with one dose of 50 mg/kg KTZ ip 0.5 h before treatment with 6.25 μg/kg calcitriol ip. G, CYP24A1 activity measured in C3H/JeH mice kidney tissues collected 24 h after treatment with a single oral dose of 2.1 μg/kg of calcitriol and mice pretreated with p.o. 200 mg/kg RC22040.5 h before calcitriol, at the time of 2.1 μg/kg of calcitriol p.o. administration and every 3.5 h thereafter for a total of five doses. CYP24A1 activity in untreated C3H/JeH kidney tissue was 0.27 fmol/min · mg protein (n = 6) was assigned with an arbitrary value of 1.

observed in kidney. The 24-OHase activity induced by ip administered calcitriol in C3H/HeJ mice treated was inhibited by both KTZ and RC2204 (Fig. 2, F and G).

Inhibition of 24-OHase activity modulates serum calcitriol PK in normal C3H/HeJ mice

The effect of the inhibition of 24-OHase activity on serum calcitriol PK was determined by measuring calcitriol levels in serum samples obtained from calcitriol alone, calcitriol + KTZ-, or calcitriol + RC2204-treated normal C3H/HeJ mice. Enhanced systemic exposure (AUC) and delayed clearance (CL/F) of calcitriol were observed in calcitriol + KTZ- and calcitriol + RC2204-treated mice when compared with mice treated with calcitriol alone (Table 1).

Calcitriol + KTZ/Dex treatment modulate serum calcium, testosterone, and creatinine levels in normal C3H/HeJ mice

Changes in calcium, creatinine, and total testosterone levels were measured in serum samples obtained from untreated, calcitriol alone, calcitriol + KTZ-, calcitriol + Dex-, KTZ + Dex-, and calcitriol + KTZ + Dex-treated C3H/HeJ mice. Serum calcium and creatinine levels were significantly increased in all calcitriol-treated groups.

TABLE 1. CYP24A1 inhibitors modulate serum calcitriol PK parameters in normal C3H/JeH mice

PK parameters	Treatment			
	Calcitriol[a]	Calcitriol + KTZ[a]	Calcitriol[b]	Calcitriol + RC2204[b]
Cmax (ng/ml)	8.73 ± 1.03	9.67 ± 3.19[c]	0.20 ± 0.02	0.80 ± 0.06[e]
AUC (ng/h · ml)	33.6 ± 3.50	45.2 ± 3.0[d]	2.43 ± 0.03	8.22 ± 0.85[e]
CL/F (ml/min)	3.75 ± 0.38	2.84 ± 0.52[d]	17.3 ± 0.22	5.20 ± 0.53[e]

[a] KTZ (50 mg/kg) and 6.25 μg/kg calcitriol both administered ip.

[b] RC2204 (200 mg/kg) and calcitriol (2.1 μg/kg), both administered p.o.

[c] Difference not significant.

[d] Significant increase in AUC and decrease in apparent clearance (CL/F) ($P < 0.05$).

[e] Significant increase in Cmax and AUC and decrease in CL/F ($P < 0.01$).

Cmax, Peak serum concentration.

TABLE 2. Treatment induced changes in C3H/JeH mice serum calcium, creatinine, and total testosterone levels

Serum parameter measured	No. treatment ($n = 5$)	Calcitriol alone ($n = 4$)	Calcitriol + KTZ ($n = 4$)	Calcitriol + Dex ($n = 4$)	KTZ + Dex ($n = 4$)	Calcitriol + KTZ + Dex ($n = 5$)	*P* (ANOVA)
Calcium (mg/dl)	8.74 ± 0.63	11.52 ± 0.60	12.64 ± 0.59	12.02 ± 0.96	8.67 ± 0.50	10.61 ± 0.81	0.0024
Creatinine (mg/dl)	2.20 ± 0.07	2.45 ± 0.10	2.62 ± 0.07	2.85 ± 0.08	1.99 ± 0.08	2.65 ± 0.07	<0.0001
Testosterone (ng/ml)	0.07 ± 0.03	0.14 ± 0.05	0.07 ± 0.27	1.21 ± 0.14	0.47 ± 0.42	1.05 ± 0.29	0.005

Results are presented as mean ± SEM.

KTZ + Dex treatment had no effect on either calcium or creatinine serum levels (Table 2). Substantial variability in serum testosterone levels was observed in all treatment groups. A trend suggesting an increase in serum testosterone levels in animal groups treated with combinations containing both calcitriol and Dex was observed (Table 2).

KTZ and Dex enhance the antiproliferative effects of calcitriol in PC3 cells

To assess the effect of KTZ inhibition on the antiproliferative effects of calcitriol, we treated PC3 cells with calcitriol, KTZ + Dex, or the combination of calcitriol + KTZ + Dex. The results of MTT assay show that antiproliferative effects were increased by combining calcitriol with KTZ/Dex when compared with either calcitriol or KTZ/Dex across all doses, as indicated by the Fraction Affected (Fig. 3A). The dose-effect data obtained for each drug alone and in combination were used to calculate the CI. For most of the dose combinations tested, a CI value of less than 1 was obtained, indicating that the interaction between calcitriol and KTZ/Dex is synergistic (Fig. 3B). In another measurement, when the combination treatment resulted in a Fraction Affected over approximately 0.2, the interaction is synergistic (Fig. 3C).

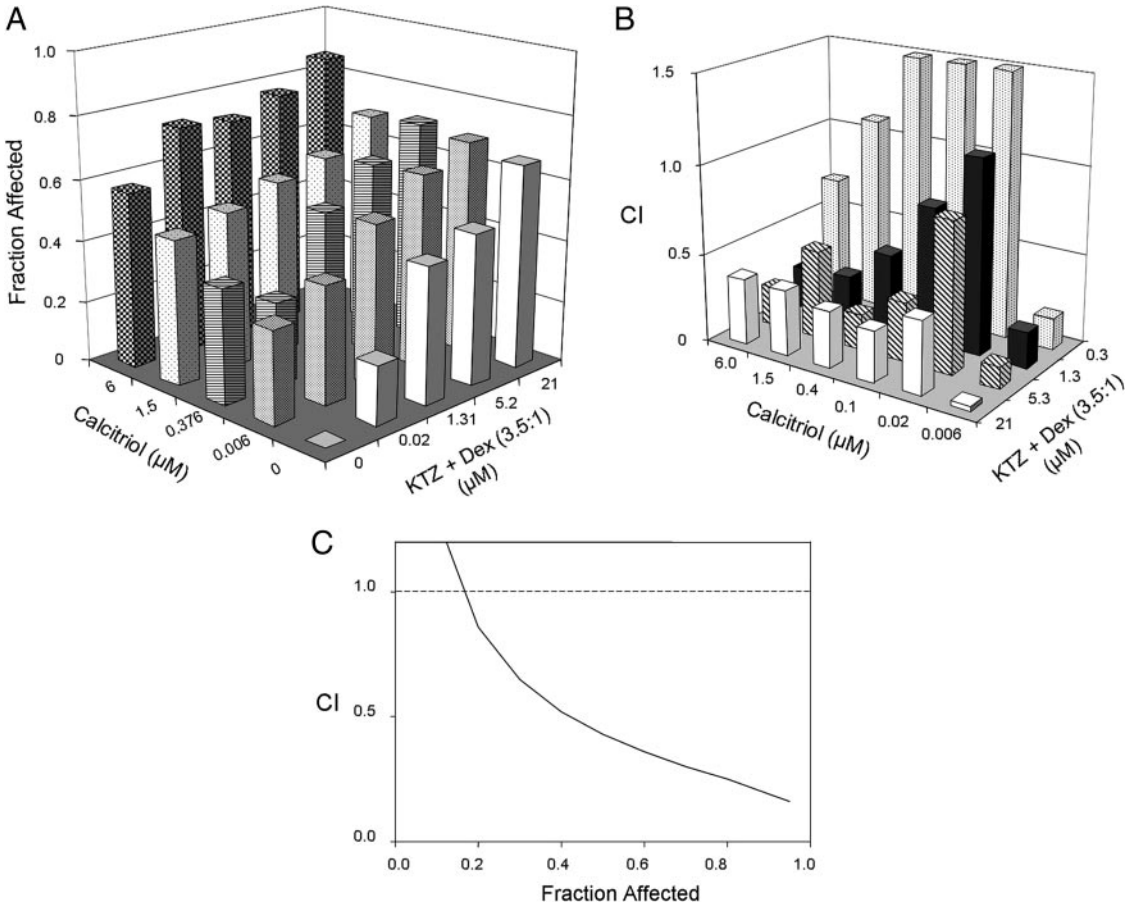

FIG. 3. Calcitriol-mediated antiproliferative effect in PC-3 cells is enhanced by KTZ/Dex. A, PC-3 cells were pretreated with 500 nM Dex for 24 h followed by varying concentrations of calcitriol and/or KTZ for 48 h. Cell growth was measured by MTT assay. B, CI of calcitriol + KTZ + Dex was determined by median dose effect analysis. CI < 1.0 is indicative of synergistic interaction. C, CI over fraction affected was determined by median dose effect analysis. CI < 1.0 is indicative of synergistic interaction.

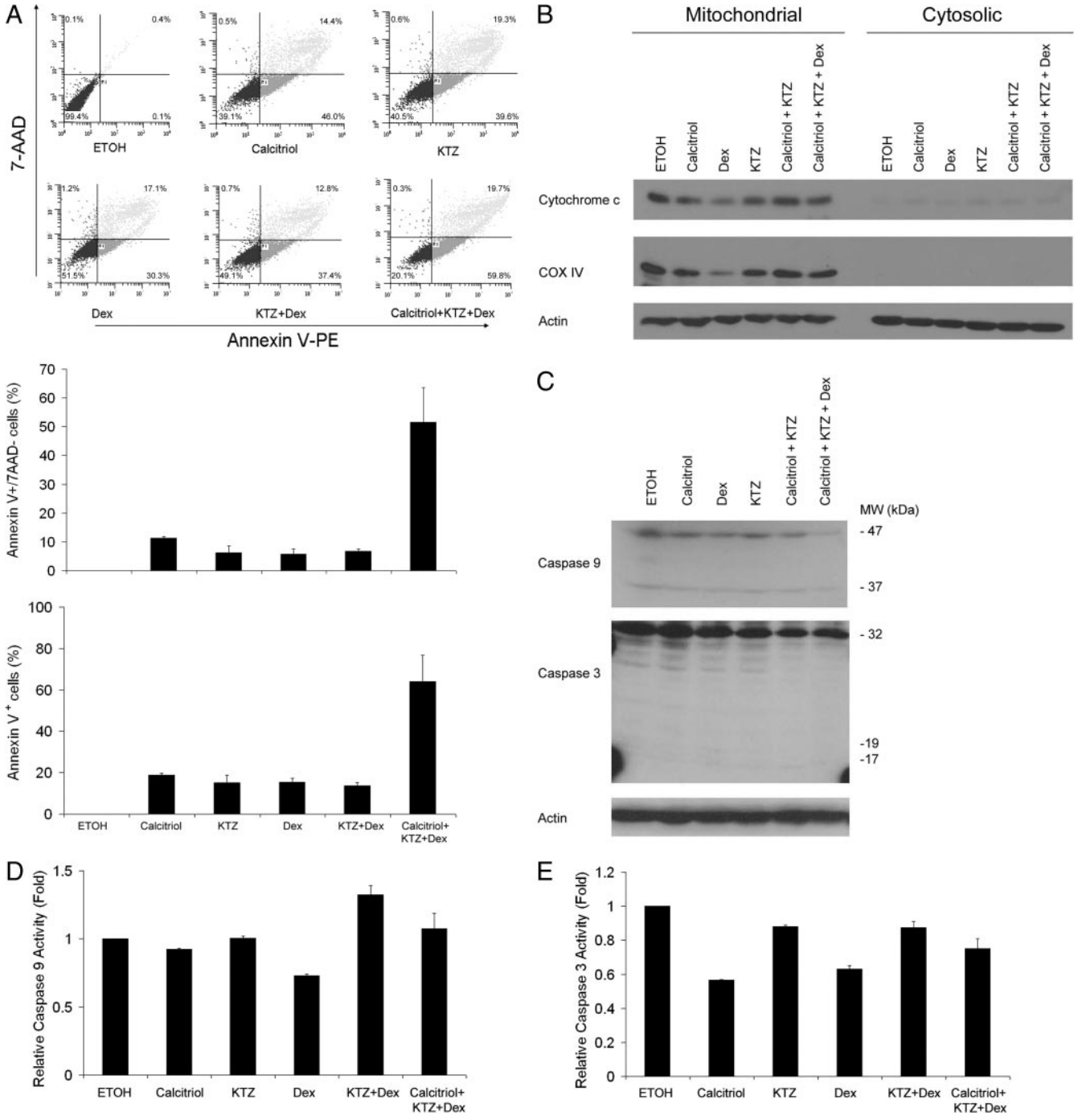

FIG. 4. Calcitriol + KTZ + Dex combination promotes apoptosis in PC-3 cells. PC3 cells were treated with EtOH, calcitriol (375 nM), Dex (375 nM), KTZ (1.316 μM), KTZ + Dex, or calcitriol + KTZ + Dex for 24 h. A, Apoptosis was assessed by annexin V/7AAD staining. Representative histograms were shown, and early (annexin V+/7AAD−) and total (annexin V+) apoptosis are presented in the *bar graph*. B, Mitochondrial and cytosolic fractions were isolated and assessed for cytochrome c release by Western blot analysis. COX IV was served as a control for mitochondrial leakage to cytosol. Actin was the loading control. C, Caspase-9 and caspase-3 cleavages were examined by Western blot analysis. D and E, Relative caspase-9 and caspase-3 activities were examined by substrate-based caspase activity assays. MW, Molecular weight.

Calcitriol, KTZ, and Dex combination induces apoptosis in PC3 cells

Induction of apoptosis in PC3 cells as a potential mechanism for the calcitriol + KTZ/Dex antiproliferative synergy was assessed by annexin V/7AAD staining. Calcitriol, KTZ, or Dex, as single agent, induced few annexin V positive cells when compared with control (Fig. 4A). The combination of KTZ and Dex did not further promote apoptosis. In contrast, a combination of calcitriol + KTZ/Dex markedly promoted apoptosis, including the early apoptosis and total apoptosis ($P < 0.05$) (Fig. 4A), indicating that KTZ/Dex promotes calcitriol-mediated apoptosis in PC3 cells.

Calcitriol and KTZ/Dex do not induce apoptosis via mitochondria-mediated pathway

To further characterize the mechanisms for the apoptosis induced in PC3 cells, the involvement of the mitochondria-dependent pathway was examined. Analysis of the mitochondrial and cytosolic compartments revealed that cytochrome *c* was not released from mitochondria to cytosol (Fig. 4B). Western blot analysis shows that the major caspases in the mitochondria-mediated apoptosis pathway, caspase-9 and caspase-3, were not activated with any treatment (Fig. 4C). Substrate-based caspase activity assays showed that caspase-9 (Fig. 4D) and caspase-3 (Fig. 4E) were not activated by any treatment. These results suggest that mitochondria-dependent apoptotic pathway is not involved in calcitriol + KTZ/Dex-induced apoptosis in PC3 cells.

Calcitriol and KTZ/Dex induce the activation of caspase-8

To examine the involvement of death receptor-mediated apoptotic pathway, the activity of caspase-8 was evaluated. Calcitriol + KTZ/Dex combination induced the cleavage of caspase-8 as assessed by Western blot analysis (Fig. 5A). Bid is a proapoptotic Bcl-2 family member, which can be cleaved to truncated Bid (tBid) by caspase-8 (36). tBid translocates to mitochondria and leads to mitochondria damage (36). Western blot analysis showed the greatest increase in tBid level after calcitriol + KTZ treatment, followed by calcitriol + KTZ/Dex combination (Fig. 5A). AIF is released from permeabilized mitochondria (37). AIF mediates caspase-independent apoptosis by translocating to the nucleus and subsequently inducing DNA fragmentation (37). Considering that the executor caspase-3 is not activated in calcitriol and KTZ/Dex-mediated apoptosis, and that the mitochondria may be damaged by increased tBid level, the involvement of AIF in this apoptosis was investigated. Calcitriol, Dex, or KTZ as single agent induced AIF in the nuclear fraction, and this induction was enhanced by the combination treatment of calcitriol and KTZ or calcitriol and KTZ/Dex, as shown by Western blot analysis of nuclear and cytosolic extracts (Fig. 5B). These results suggest that calcitriol and KTZ/Dex-mediated apoptosis may involve AIF-induced, caspase-independent pathway.

KTZ and Dex enhance antitumor effects of calcitriol in a human PC3 prostate xenograft model

To investigate whether reduced 24-OHase activity potentiates calcitriol antitumor activity *in vivo*, we employed an *in vivo* excision clonogenic assay, which is an indication of antitumor effects *in vivo* (6). PC3 prostate xenograft tumor-bearing nude mice were treated with saline,

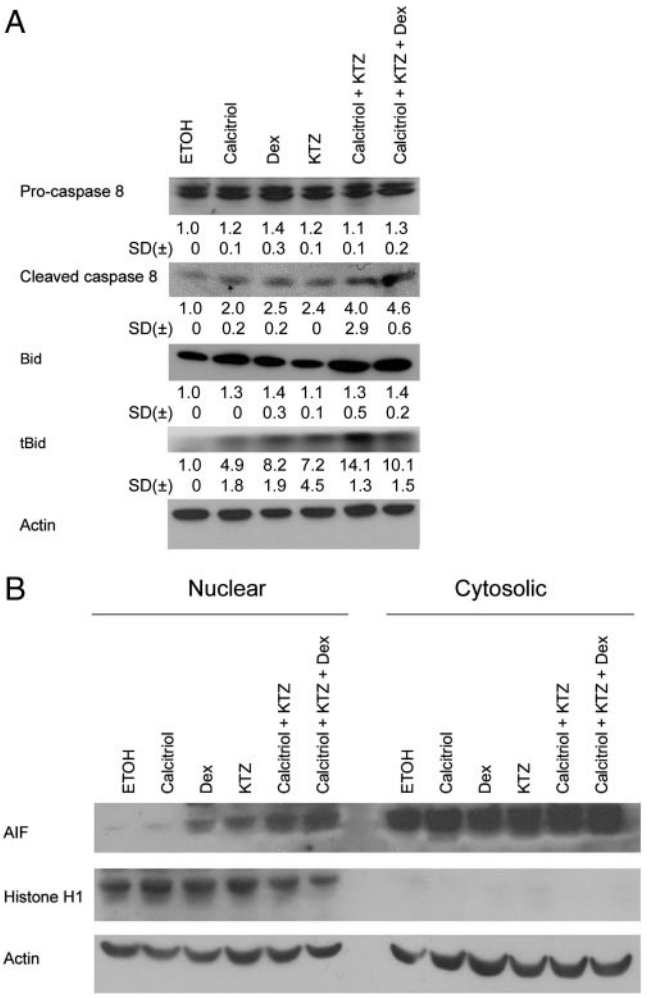

FIG. 5. Calcitriol + KTZ + Dex-induced apoptosis involves AIF translocation. PC3 cells were treated with EtOH, calcitriol (375 nM), Dex (375 nM), KTZ (1.316 μM), KTZ + Dex, or calcitriol + KTZ + Dex for 24 h. A, Caspase-8 cleavage and the levels of Bid and tBid were examined by Western blot analyses. Densitometry analysis was conducted, and the densities of the bands were presented as the average ± SD of the fold changes normalized to the ETOH control treatment (1.0). B, Nuclear and cytosolic fractions were isolated and examined for AIF levels by Western blot analysis. Histone H1 was a control for nuclear fraction. Actin was the loading control.

calcitriol, KTZ, Dex, KTZ + Dex, or the three drug combination (calcitriol + KTZ + Dex), and the tumors were harvested for clonogenic assay. The combination of calcitriol, KTZ, and Dex resulted in greater decrease in surviving fraction than any single agent or KTZ + Dex treatment ($P < 0.05$) (Fig. 6A). We further examined the effects of calcitriol and KTZ/Dex on tumor growth in the same model. We observed greater tumor growth delay in animals treated with the three-drug combination than with any single agent or KTZ + Dex combination (Fig. 6B). To study whether the *in vitro* observations of the effects of calcitriol and KTZ + Dex are consistent *in vivo*, tumor tissues were harvested at the end of the treatment, and immunohistochemistry studies were performed with the proliferation marker Ki-67 and apoptosis marker cleaved

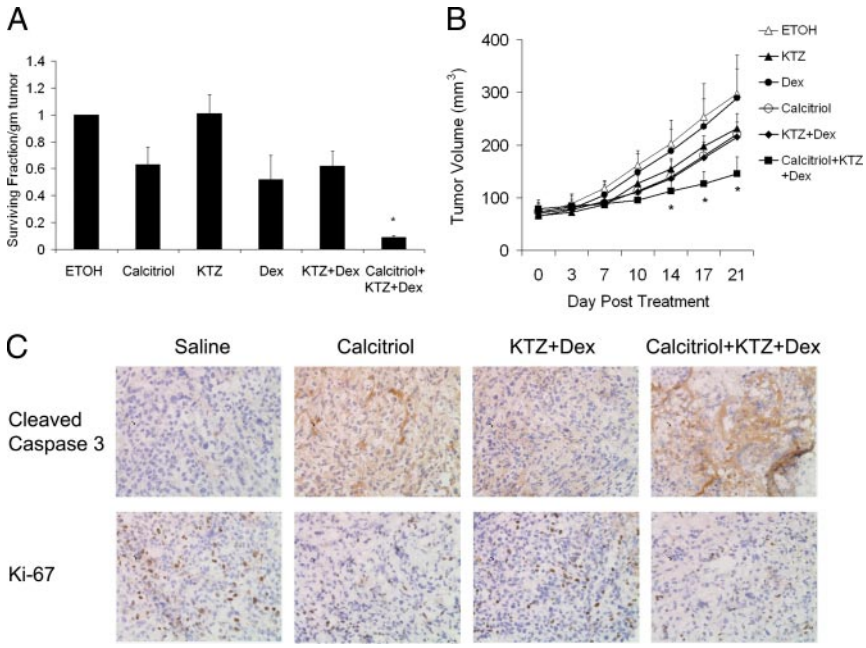

FIG. 6. KTZ/Dex potentiates calcitriol antitumor activity *in vivo*. PC3 cells were inoculated sc into nude mice. When the tumors were palpable, the mice were treated with various combinations of calcitriol, Dex, and/or KTZ for 3 wk (see details in *Materials and Methods*). A, The *in vivo* clonogenic assay was performed 24 h after the last treatment. *, $P < 0.01$. B, Tumor growth inhibition was assessed by measuring tumor size with a caliper three times per week. Tumor volumes were calculated by (length × width^2)/2 and expressed as a fraction of the pretreatment at the time of the first treatment. *, $P < 0.01$. C, Immunohistochemical detection of Ki-67 and cleaved caspase-3 in PC3 xenograft tumor tissues. PC3 tumors were harvested after the treatment, and tissues were stained with antibodies for Ki-67 and cleaved caspase-3.

caspase-3. The results showed that control saline group did not have positive cleaved caspase-3 staining, whereas calcitriol treatment alone induced caspase-3 cleavage in the tumor tissue (Fig. 6C). KTZ + Dex induced modest caspase-3 cleavage, and importantly, the combination of calcitriol and KTZ + Dex further enhanced caspase-3 cleavage (Fig. 6C). On the other hand, saline-treated tumor tissue had strong Ki-67 staining, calcitriol-reduced Ki-67 staining, whereas KTZ + Dex did not affect its staining (Fig. 6C). The combination treatment further reduced Ki-67 staining. These findings indicate that calcitriol inhibits PC3 tumor proliferation and promotes apoptosis, and these effects are enhanced in combination with KTZ + Dex.

Discussion

Calcitriol exerts growth inhibitory effects in a number of cancer cells. However, prostate cancer PC3 cells are insensitive to calcitriol treatment *in vitro*. This prompted us to study whether calcitriol metabolizing enzyme CYP24A1 plays a role in this resistance. Our study indicates that calcitriol induces CYP24A1 protein expression and enzyme activity. There is also synergistic enhancement of antiproliferative effects of calcitriol when used in combination with KTZ, a broad inhibitor of cytochrome P450 enzymes, including CYP24A1. The results of our PK and pharmacodynamic studies indicate effective *in vivo* inhibition of 24-OHase activity in kidney, the major calcitriol-inactivating organ, a 30% enhancement of systemic calcitriol exposure and delayed serum calcitriol clearance in normal mice treated with the combination of calcitriol + KTZ. Although a direct cause and effect relationship between the pharmacologic inhibition of CYP24A1 activity and the increase in systemic calcitriol exposure was not established in this study, similar serum calcitriol PK characteristics were reported in CYP24A1 knockout mouse models (38). The modulation of calcitriol PK by KTZ is, however, relatively inferior when compared with results in CYP24A1 knockout mice models.

To investigate the role of apoptosis in the enhanced antiproliferative effects of calcitriol by KTZ/Dex, we examined the activation of caspase-dependent and caspase-independent apoptosis pathways. Calcitriol is known to induce apoptosis in a number of human cancer cell lines. We previously demonstrated that calcitriol enhanced paclitaxel-mediated growth inhibition and apoptosis of in PC3 cells (29). Apoptosis, as assessed by poly(ADP-ribose) polymerase cleavage, was not observed in calcitriol alone-treated cells (29). This study indicates that the combination of calcitriol + KTZ + Dex in PC3 cells induces substantial apoptosis, detected by quantitative annexin V staining. These *in vitro* observations were confirmed by the *in vivo* results of our immunohistochemistry studies of PC3 prostate xenograft tumor biopsies that indicated an increase in antiproliferative and apoptotic effects of calcitriol + KTZ + Dex combination compared with calcitriol alone treatment.

Calcitriol + KTZ + Dex-induced apoptosis is not mediated by a mitochondria-dependent pathway, because cytochrome *c* release from mitochondria is not observed and neither caspase-3 nor caspase-9 were activated. These observations suggest that a caspase-independent apoptotic pathway is involved. Our results support the nuclear translocation of AIF, which is an important mediator of caspase-independent pathway. Together, our data suggest calcitriol and KTZ/Dex induce apoptosis through a non-

classic apoptotic pathway, the AIF-mediated and caspase-independent pathway. Previously reported mechanisms of calcitriol-induced apoptosis have included: regulation of the expression of Bcl-2 and inhibitor of apoptosis family proteins (39, 40), altered expression of proteins in p38 MAPK, MAPK kinase, and ERK pathways (41), activation of calcium-dependent cysteine proteases, mitochondria-dependent caspase pathway, and p53-independent mechanisms (42–45). To the best of our knowledge, this is first report documenting the role of the AIF-mediated and caspase-independent pathway in calcitriol induced apoptosis.

The *in vivo* studies in the PC3 xenograft model demonstrate that the calcitriol inhibited tumor growth as a single agent, and more importantly, the combination of calcitriol and KTZ/Dex significantly reduced the tumor growth. Based on these results, we suggest that the enhancement of the calcitriol antitumor activity by KTZ/Dex combination could be mediated by multiple events. First, the inhibition of CYP24A1 activity diminishes the degradation of calcitriol, thus increasing target tissue and systemic calcitriol levels. Second, the resulting high intracellular calcitriol in combination with KTZ/Dex kills tumor cells by AIF-induced, caspase-independent apoptotic pathway. Third, antiproliferation and apoptosis of tumor cells in xenograft models of prostate cancer could also be modulated by calcitriol + KTZ + Dex treatment-induced changes in androgen levels. However, the immediate changes in serum total testosterone levels observed in this study were within the normal mouse range (0.13–1.69 ng/ml) (46). The long-term effect of calcitriol + KTZ + Dex treatment on androgen levels has not been studied.

To the best of our knowledge, this is the first report to show the *in vivo* pharmacological inhibition of CYP24A1 activity and the enhancement of the calcitriol antitumor activity by KTZ/Dex in a human prostate cancer PC3 xenograft model. However, the clinical utility of this combination remains a challenge for the following questions. First, what is the advantage of using calcitriol in combination with CYP24A1 inhibitors *vs.* giving high-dose calcitriol alone? A theoretical advantage of using CYP24A1 inhibitors in combination with calcitriol is based on the overexpression of CYP24A1 in tumor tissues compared with normal tissues. Therefore, inhibition of this enzyme could result in higher calcitriol levels in target tumor tissue, thus enhancing the selectivity and tumor cell kill. Second, what additional side effects related to the use of CYP24A1 inhibitors would be expected? Although clinical studies designed to investigate the use of CYP24A1 inhibitors in combination with vitamin D_3-based therapies are justified, these studies will require the identification of selective and potent CYP24A1 inhibitors with favorable PK and safety profiles.

In summary, our data demonstrate *in vitro* and *in vivo* inhibition of CYP24A1 activity by KTZ. The inhibition of CYP24A1 activity by KTZ is associated with increases in systemic calcitriol exposure, together with the activation of AIF-induced apoptotic pathway by a combination of calcitriol + KTZ + Dex, contributes to the enhancement of calcitriol antitumor effects in preclinical models.

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