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Vitamin D analogues targeting CYP24 in chronic kidney disease☆

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ABSTRACT

The cytochrome P450 enzyme 24-hydroxylase (CYP24) plays a critical role in regulating levels of vitamin D hormone. Aberrant expression of CYP24 has been implicated in vitamin D insufficiency and resistance to vitamin D therapy. We have demonstrated amplified CYP24 expression in uremic rats, suggesting that CYP24 has an etiological role in vitamin D insufficiency commonly associated with chronic kidney disease (CKD). We have designed two new analogues of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), namely CTA091 and CTA018/MT2832, which are potent inhibitors of CYP24. *In vitro* studies with CTA091 show that it enhances the potency of 1α,25$(OH)_2D_3$. *In vivo* studies demonstrate that CTA091 decreases serum intact parathyroid hormone (iPTH) levels and increases circulating 1α,25$(OH)_2D_3$. CTA091 increases both C_{max} and AUC of co-administered 1α,25$(OH)_2D_3$. These studies indicate that CYP24 inhibition can increase cellular responsiveness to vitamin D hormone and potentiate vitamin D therapy. CTA018/MT2832 differs from CTA091 in that it also has the ability to activate vitamin D receptor-mediated transcription. CTA018/MT2832 effectively suppresses elevated iPTH secretion at doses which do not affect serum calcium or phosphorus levels in a rodent model of CKD. Studies with both new analogues underscore the potential utility of CYP24 inhibition in the treatment of secondary hyperparathyroidism in CKD.

1. Introduction

Chronic kidney disease (CKD) is associated with a progressive reduction of circulating 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and 25-hydroxyvitamin D_3 (25$(OH)D_3$), and a concomitant development of secondary hyperparathyroidism (SHPT), a disorder which is characterized by elevated secretion of intact parathyroid hormone (iPTH) and parathyroid hyperplasia [1,2]. Vitamin D hormone analogues are commonly used to treat SHPT, but their effectiveness is often limited by episodic hypercalcemia. Such analogues have been designed to target the vitamin D receptor (VDR). However, other intracellular factors besides VDR affinity may influence the efficacy and safety of vitamin D hormone replacement therapies.

The effectiveness of currently used vitamin D analogues in initiating transcriptional regulation of targeted genes is determined largely, if not entirely, by the ability of these agents to bind the VDR. As most tissues express VDR, specific tissue responses to these therapies are governed by a number of intracellular factors, including extra-renal 1α-hydroxylase (CYP27B1), which permits localized synthesis of additional 1α,25$(OH)_2D_3$, and the cytochrome P450 enzyme 24-hydroxylase (CYP24), which hydroxylates, and thereby inactivates 1α,25$(OH)_2D_3$ and 25$(OH)D_3$ and administered analogues [3,4]. Normally, expression of CYP24 is elevated as intracellular 1α,25$(OH)_2D_3$ levels rise to limit exposure, thereby preventing toxicity. Deletion of CYP24 in mice results in a greatly diminished clearance rate for 1α,25$(OH)_2D_3$, resulting in increased vitamin D hypersensitivity and soft tissue calcification [5,6], providing strong evidence that local CYP24 expression is a key determinant of tissue responsiveness to vitamin D. Furthermore, abnormally elevated CYP24 in certain disease states, such as hypophosphatemia [7,8] and certain types of cancer [9–11], associates with decreased vitamin D status and with vitamin D resistance. Recent evidence also suggests that CYP24 expression may be dysregulated in uremic kidneys [12,13].

In 1987, the DeLuca group reported a 25-methyl ether version of the natural hormone 1α,25$(OH)_2D_3$ (Fig. 1A) that retained most of the pro-differentiation activity of 1α,25$(OH)_2D_3$ [14]. This led to the hypothesis that the Lewis basic 25-oxygen atom of the methyl ether served as a hydrogen-bond acceptor in the binding pocket of the VDR [14]. In light of this information, we reasoned that

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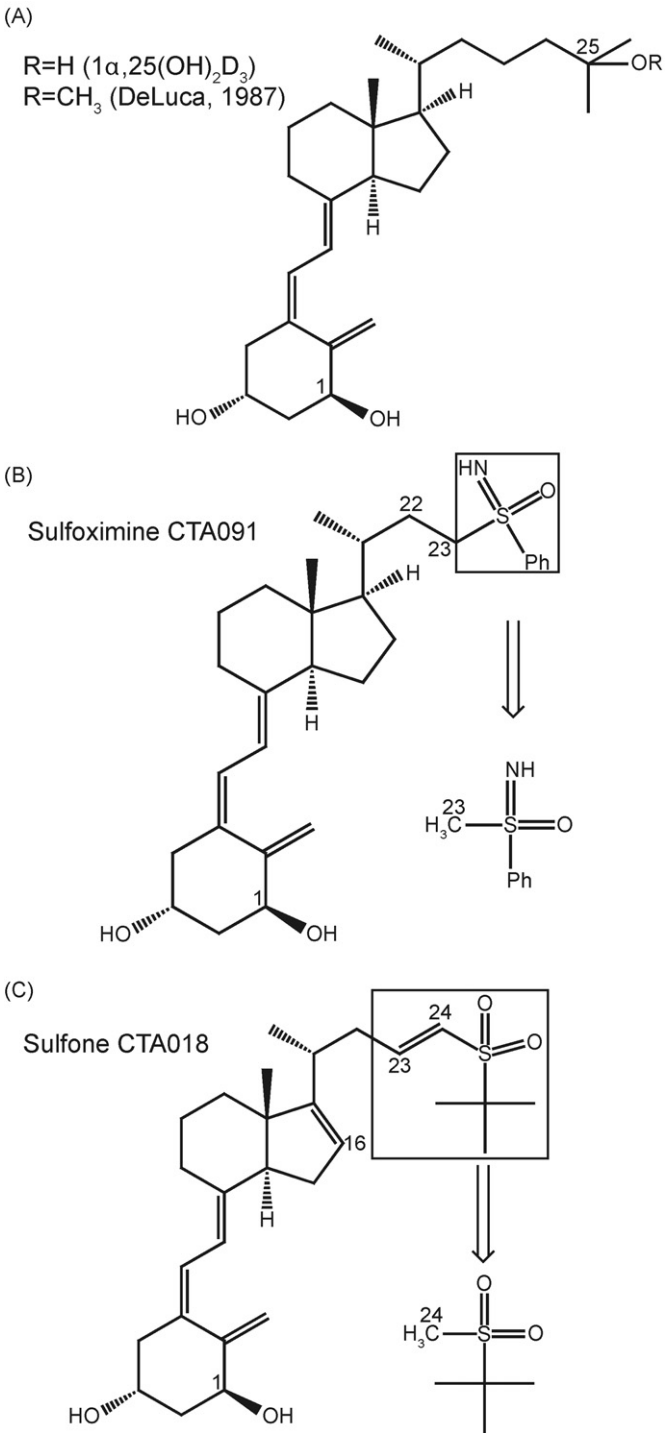


Fig. 1. Chemical structures of $1\alpha,25(OH)_2D_3$, CTA091 and CTA018/MT2832.

other hydrogen-bond acceptors at the terminus of the C,D-ring side chain would be worth examining. We prepared sulfoximine analogues of $1\alpha,25(OH)_2D_3$ in which the sulfoximine functional group could act in the VDR binding pocket as a hydrogen-bond donor via its NH unit and/or as a hydrogen-bond acceptor via its S=O unit. Moreover, we also prepared a series of side chain sulfone analogues capable of acting as side chain hydrogen-bond acceptors [15]. Inspired by seminal Hoffman–LaRoche studies [16], we incorporated a 16,23-diene unit into our designer sulfones. We identified two promising analogues: the sulfoximine MK-24(*S*)-*S*(O)(NH)-Ph-1 (CTA091), which is a potent, selective and non-calcemic inhibitor of CYP24 [17] (Fig. 1B), and the sulfone GHP-GH-16,23-diene-25S02-1 (CTA018/MT2832), which is a potent and low-calcemic inhibitor of CYP24, as well as a potent activator of VDR-mediated transcription [15] (Fig. 1C). In this paper, we investigated the biological properties of both CTA091 and CTA018/MT2832 *in vitro* and *in vivo*, using normal and adenine-induced uremic rats.

2. Materials and methods

2.1. Cell culture

V79-CYP24 cells were grown in DMEM, 5% FBS and 100 μg/mL Hygromycin B for selection of CYP24-transfected cells. U937 cells were grown in 4 + RPMI media containing 2 g/L sodium bicarbonate and glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% fetal calf serum. For transcription activation studies, U937 were stimulated with PMA (10 mg/mL PMA for 24 h). Culture media was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. CYP24 enzyme activity assay

V79-CYP24 cells were plated in 100 μL of DMEM media containing 1% BSA and treated with varying amounts of CTA091 or CTA018/MT2832 (10^{-6}–10^{-10} M). After 30 min [$1\beta^3$H]-$1\alpha,25(OH)_2D_3$ (radioactivity 40 Ci/mM; Cytochroma Inc., Markham, ON, Canada) and DPPD were added to a final concentration of 10 and 100 nM, respectively. Following a 2 h incubation at 37 °C, the reaction was terminated with methanol (250 μL). The water soluble product was recovered by Bligh–Dyer extraction. Aliquots of the upper aqueous phase were measured using a scintillation counter. The radioactivity of the aqueous phase was used as a measure of conversion. Ketoconazole (10^{-5}–10^{-9} M; Sigma–Aldrich, St. Louis, MO, USA) was used as a positive control. Inhibition of CYP24 activity was calculated relative to the highest conversion rate control, with the non-specific background from samples with complete inhibition subtracted (10 μM ketoconazole).

2.3. Real-time PCR

PMA stimulated-U937 cells were treated with 1, 10 or 100 μM of $1\alpha,25(OH)_2D_3$ (Sigma-Aldrich) and CTA091 or CTA018/MT2832 for 6 h at 37 °C. Media was removed and RNA was isolated using 1 mL of TRIzol® reagent. cDNA was synthesized from 1 μg of RNA using the Thermoscript™ RT-PCR System Kit (Invitrogen). Quantitative real-time PCR (RT-qPCR) was used to detect the CYP24 mRNA using Taqman® gene expression assays purchased from Applied Biosystems (ABI; Foster City, CA, USA) using the following ID numbers: human GAPDH (Hs99999905_m1) and human CYP24 (Hs00167999_m1). RT-qPCR results were analyzed using sequence detection system software V1.0 (ABI). Gene expression levels were calculated using the comparative C_T method, and normalized to GAPDH expression levels (% relative induction).

2.4. Ligand binding assay

Human recombinant VDR (1 pmol/reaction; Biomol, Plymouth Meeting, PA, USA) was prepared in binding buffer containing 50 mM Tris–HCl pH 7.4, 1.5 mM EDTA, 300 mL KCl, 5 mg/mL gelatin and 10 mM DTT, and preincubated with $1\alpha,25(OH)_2D_3$ (10^{-6}–10^{-10} M), CTA091 or CTA018/MT2832 (10^{-6}–10^{-10} M) for 1 h at room temperature. [^{3}H]$1\alpha,25(OH)_2D_3$ (25 nM, 20,000 cpm, Perkin Elmer, Boston, MA, USA) was added to the solution and incubated for 1 h at room temperature. The unbound radioactivity was removed by incubation with 100 mL of charcoal/dextran solution

on ice for 30 min and then pelleted by centrifugation 2000 rpm at 4 °C for 10 min. 100 μL of aqueous phase was counted by liquid scintillation. Unlabelled 1α,25(OH)$_2$D$_3$ was used as a control. The VDR binding of CTA091 or CTA018/MT2832 was calculated relative to 1α,25(OH)$_2$D$_3$, referred to as 1.

2.5. Serum biochemistry

Adult male Sprague Dawley rats (6–8 weeks of age) were purchased from Hilltop Lab Animals Inc. (Scottdale, PA, USA). To determine the effects of CTA091, rats were administered a single intravenous injection of vehicle (propylene glycol:saline:ethanol (30:50:20, v/v/v)), CTA091 (500 μg/kg), 1α,25(OH)$_2$D$_3$ (16 μg/kg) or 1α,25(OH)$_2$D$_3$ + CTA091. Blood samples were collected at 0, 3, 6, 12 and 24 h via a jugular catheter. To assess the effects of CTA018/MT2832 treatment in uremic rats, animals were fed a standard diet (Certified Rodent Diet 5002) alone or containing 0.75% adenine to induce uremia. After 4 weeks on these diets, uremic and normal animals were treated intravenously 3 times per week with CTA018/MT2832 (0.05, 0.10, 0.25, 0.50, 1.0 and 6.0 μg/kg) or vehicle (same as above) for 4 weeks. Serum or plasma was collected at 24 h post final injection. Serum 1α,25(OH)$_2$D$_3$ was extracted using Accubond II ODS-C18 100 mg, 1 mL SPE cartridges (Agilent Technologies, Palo Alto, CA, USA). The collected fractions were dried under a steady stream of nitrogen gas and residues reconstituted in 50 μL of methanol/H$_2$O (80/20, v/v) and analyzed using LC–MS/MS (Waters Alliance HPLC-Waters Quattro Ultima Mass Spectrometer). Serum iPTH levels were measured for CTA091-treated rats using the Milliplex^{TM} Rat Bone Panel 3 bead-based immunoassay (Millipore, Billerica, MA, USA) in combination with

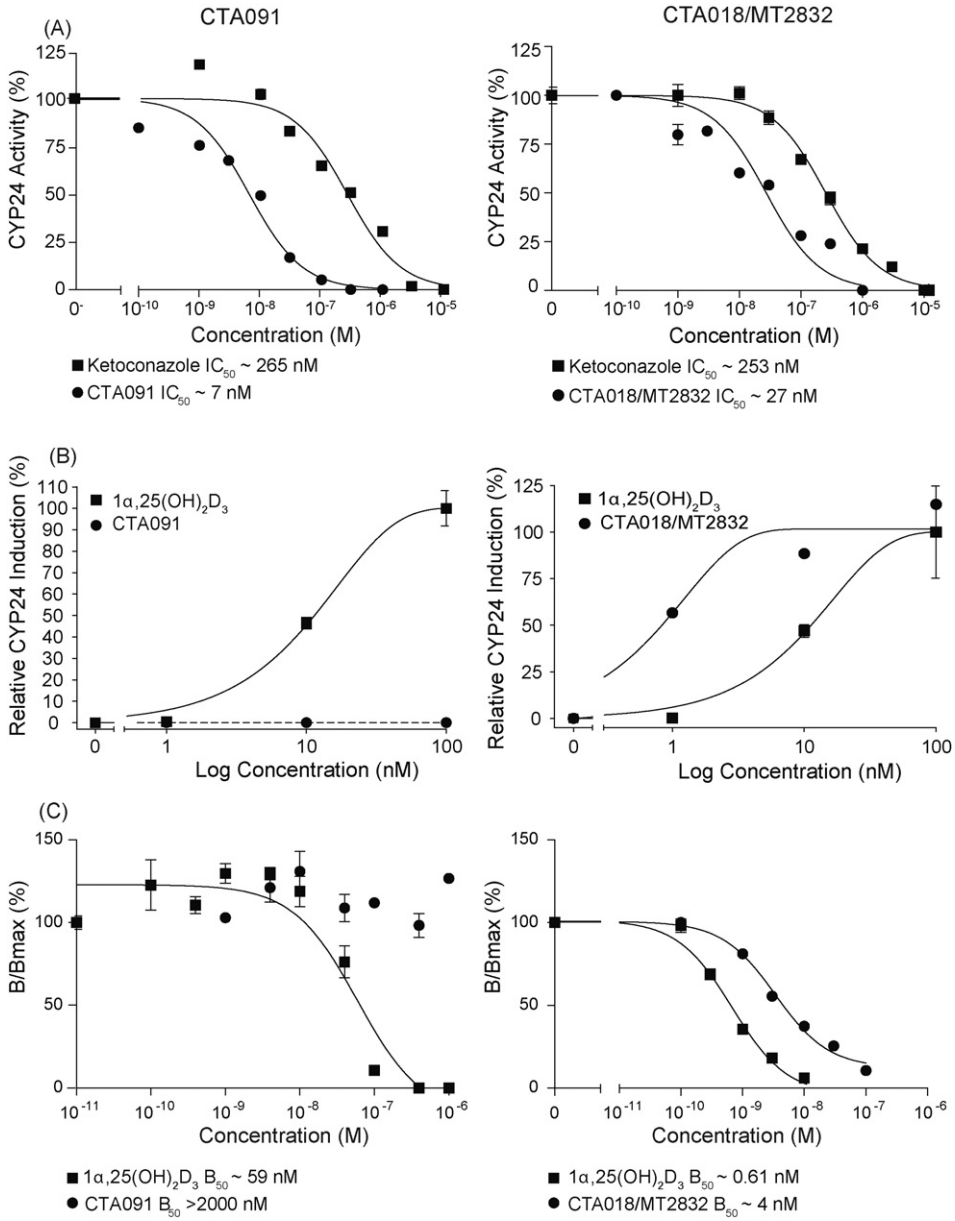
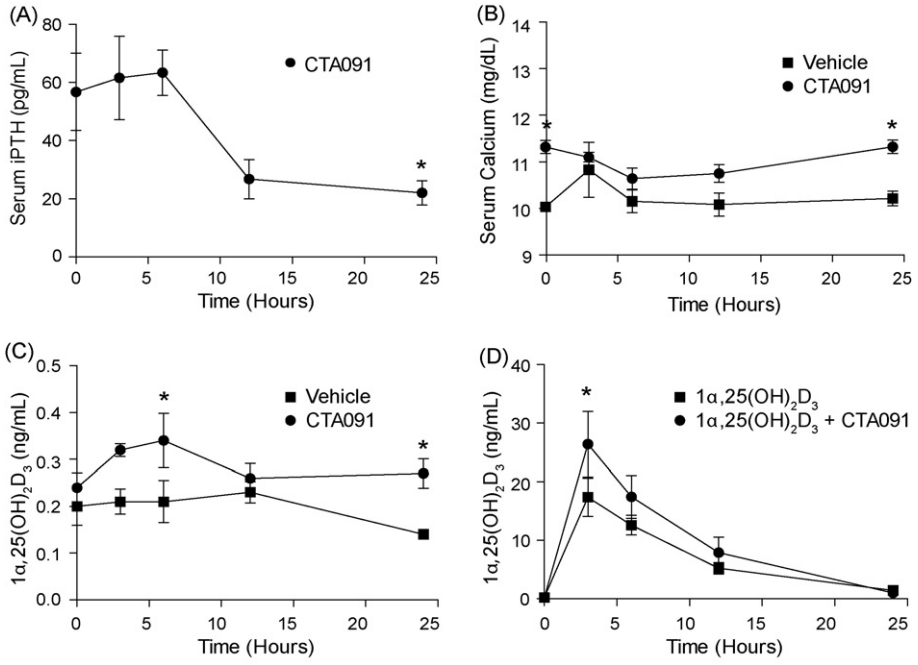


Fig. 2. CTA091 and CTA018/MT2832 as CYP24 inhibitors and VDR agonists. (A) Relative values of CYP24 activity (%) are plotted as a function of inhibitor concentration: ketoconazole (■; 10^{-5}–10^{-9}) M, CTA091 (●; *left panel*; 10^{-6}–10^{-10} M) and CTA018/MT2832 (●; *right panel*; 10^{-6}–10^{-10} M). Data are presented as mean ± SD. IC_{50} values for ketoconazole and each analogue are shown below each figure. (B) Relative induction of CYP24 mRNA (%) caused by 1α,25(OH)$_2$D$_3$ (■) CTA091 (●; *left panel*) and CTA018/MT2832 (●; *right panel*) at 1, 10 and 100 μM. Data are presented as mean ± SD. (C) Percent B/B_{max} for 1α,25(OH)$_2$D$_3$ (■) CTA091 (●; *left panel*) and CTA018/MT2832 (●; *right panel*) are plotted as a function of increasing concentration (10^{-6}–10^{-10} M). Data are presented as mean ± SD. B_{50} values for 1α,25(OH)$_2$D$_3$ and each analogue are shown below each figure.



(E)

Treatment	C_{max} (ng/mL)	AUC_{0-24} (ng.hr/mL)
	$1\alpha,25(OH)_2D_3$	
$1\alpha,25(OH)_2D_3$	17.4	175.3
CTA091+$1\alpha,25(OH)_2D_3$	26.2	241.4

Fig. 3. Pharmacodynamic effects of CTA091 in normal rats. Changes in (A) serum iPTH, (B) serum calcium, and (C) serum $1\alpha,25(OH)_2D_3$ following a single injection of vehicle (■) CTA091 (●; 500 μg/kg) or (D) $1\alpha,25(OH)_2D_3$ (■; 16 μg/kg) with or without CTA091. (E) Pharmacokinetic properties of $1\alpha,25(OH)_2D_3$ (16 μg/kg) co-administered with CTA091 (500 μg/kg). Area under the curve (AUC) was measured from 0 to 24 h and C_{max} represents the peak concentration detected. Data are presented as mean ± SEM. *Significantly different from time zero or vehicle control.

a BioPlex 200 suspension array system (Bio-Rad, Hercules, CA, USA). Plasma iPTH levels measured for CTA018/MT2832-treated rats were determined using the rat iPTH ELISA kit (Immutopics, San Clemente, CA, USA). Serum phosphorus and calcium were determined using an o-cresolphthalein complexone-based assay and an ammonium molybdate-based assay (Pointe Scientific, Canton, MI, USA), respectively. ANOVA (one- or two-way) and Bonferroni Multiple Comparison post-test were used to determine statistical significance set at $p < 0.05$.

3. Results

3.1. CTA091 and CTA018/MT2832 as CYP24 inhibitors

CTA091 and CTA018/MT2832 were screened and selected based on their abilities to inhibit CYP24 activity *in vitro*. CTA091 inhibited the CYP24 enzyme with an IC_{50} of 6.5 ± 0.2 nM, and was about 40 times more potent than the non-selective CYP24 inhibitor ketoconazole (IC_{50} 265 ± 9 nM) (Fig. 2A: *left panel*). CTA018/MT2832 inhibited CYP24 with an IC_{50} 27 ± 6 nM, about 10 times more potent than the non-selective CYP24 inhibitor ketoconazole (253 ± 20 nM) (Fig. 2A: *right panel*).

3.2. CTA091 and CTA018/MT2832 as VDR agonists

Induction of CYP24 mRNA expression was used as a marker for the ability of these two vitamin D analogues to induce VDR-mediated gene expression *in vitro*. CTA091 did not induce CYP24 mRNA at any concentration tested (1, 10, 100 nM) (Fig. 2B: *left panel*). In contrast, CTA018/MT2832-induced VDR-mediated transcription of CYP24 mRNA was 58.5 ± 10.9% (1 nM) and 92.1 ± 14.5% (10 nM) greater than $1\alpha,25(OH)_2D_3$ (2.6 ± 1.0% (1 nM) and 59.9 ± 14.7% (10 nM)) (Fig. 2B: *right panel*). CTA091 did not show any measurable affinity for the VDR using an *in vitro* competitive binding assay (Fig. 2C: *left panel*), whereas CTA018/MT2832 bound to the VDR with an affinity that was about 15-fold lower than $1\alpha,25(OH)_2D_3$ (Fig. 2C: *right panel*).

3.3. Pharmacodynamic effects of CTA091 in normal rats

A single injection of CTA091 (500 μg/kg) in normal rats suppressed serum iPTH levels by more than 50% in the absence of any significant changes to serum calcium (Fig. 3A and B). Mean serum iPTH levels decreased from a pre-treatment baseline of 56.7 ± 13.3 pg/mL to 22.1 ± 4.2 pg/mL ($p < 0.05$) at 24 h post-dose in response to a 1.5-fold increase in mean circulating $1\alpha,25(OH)_2D_3$ at 6 h (0.34 ± 0.06 ng/mL) versus the pre-treatment baseline of 0.21 ± 0.04 ng/mL ($p < 0.05$) (Fig. 3C). Co-injection of CTA091 (500 μg/kg) and $1\alpha,25(OH)_2D_3$ (16 μg/kg) to normal rats increased the observed peak concentration (C_{max}) and area under the curve (AUC) of circulating $1\alpha,25(OH)_2D_3$ by 51% and 38%, respectively, compared to the injection of $1\alpha,25(OH)_2D_3$ (16 μg/kg) alone (Fig. 3D and E). Mean serum levels of $1\alpha,25(OH)_2D_3$ increased to 26.4 ± 2.5 ng/mL at 3 h after co-administration of the two ana-

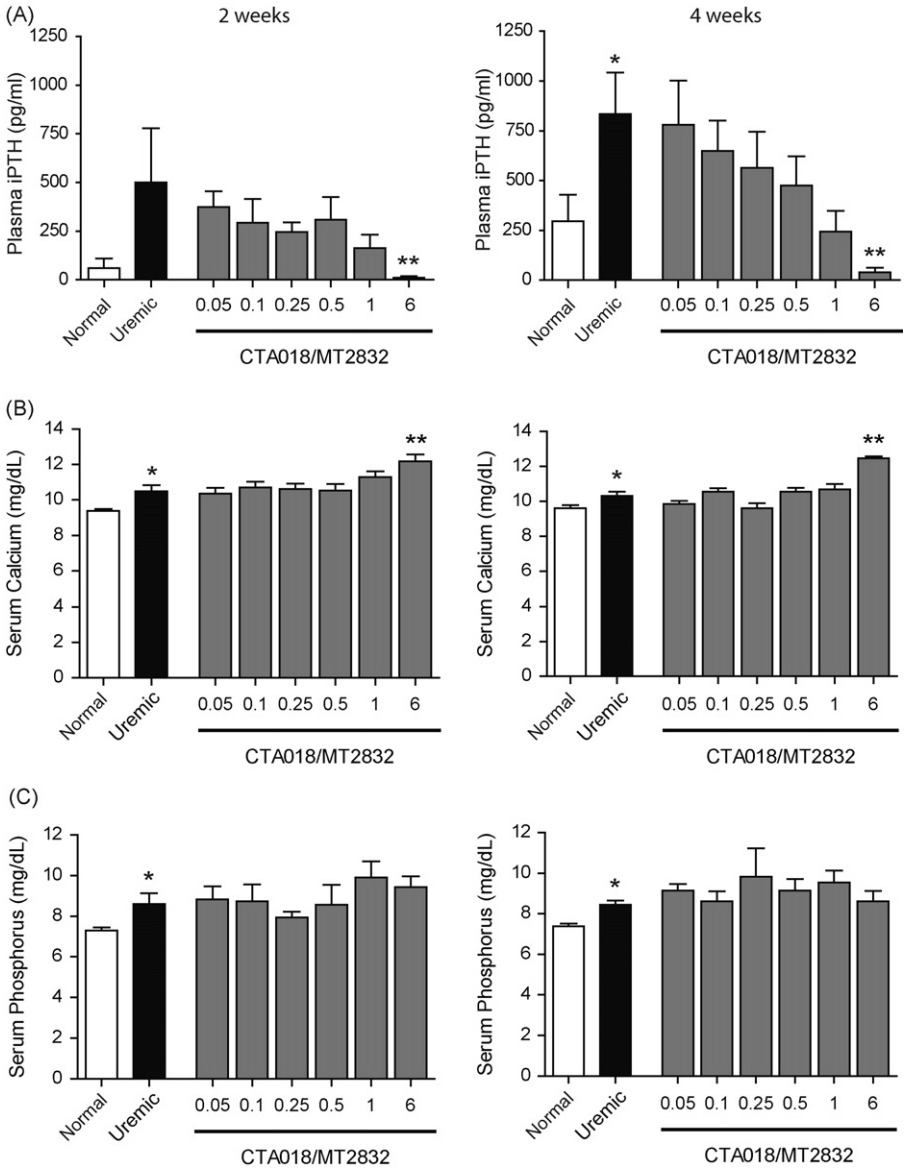


Fig. 4. Therapeutic effects of CTA018/MT2832 in uremic rats. (A) Plasma iPTH; (B) calcium; (C) phosphorus in normal (*clear bar*), uremic (*black bar*), and uremic animals exposed to varying concentrations of CTA018 (0.05, 0.1, 0.25, 0.50, 1.0 and 6.0 μg/kg; *grey bar*) are presented. Serum parameters were measured at 2 and 4 weeks with CTA018/MT2832 treatment. Data are presented as mean ± SEM. *Significant difference from normal animals. **Significant difference from uremic animals.

logues versus only 17.4 ± 3.3 ng/mL ($p < 0.01$) after administration of the hormone alone (Fig. 3D). The corresponding AUC increased to 241.1 ng h/mL versus 175.3 ng h/mL ($p < 0.01$) (Fig. 3E).

3.4. Therapeutic effects of CTA018/MT2832 in uremic rats

CTA018/MT2832 was administered intravenously 3 times per week for 4 weeks to examine its effects on plasma levels of iPTH, and serum levels of calcium and phosphorus in uremic rats. Plasma and serum levels were measured at 2 and 4 weeks. Plasma iPTH levels in adenine-induced uremia were elevated nearly 8-fold to 500 ± 277 pg/mL at 2 weeks (versus 60 ± 48 pg/mL for normal controls) and about 3-fold to 833 ± 208 pg/mL (versus 296 ± 133 pg/mL for normal controls; $p < 0.05$) at 4 weeks in the absence of treatment, but were progressively reduced with increasing doses of CTA018/MT2832 (Fig. 4A). At a dose of 6.0 μg/kg, CTA018/MT2832 significantly reduced mean iPTH levels to 11 ± 7.2 pg/mL ($p < 0.05$) and 39 ± 23 pg/mL ($p < 0.05$) at 2 and 4 weeks, respectively. Both serum calcium and phosphorus were elevated in uremia to 11 ± 0.3 mg/dL ($p < 0.05$) and 8.6 ± 0.5 mg/dL ($p < 0.05$) at 2 weeks, respectively, compared to normal controls (calcium = 9.4 ± 0.1 mg/dL; phosphorus = 7.3 ± 0.1 mg/dL) (Fig. 4B and C). At 4 weeks, calcium and phosphorus were increased in uremia to 10 ± 0.2 mg/dL ($p < 0.05$) and 8.5 ± 0.2 ($p < 0.05$), respectively, compared to normal controls (calcium = 9.6 ± 0.2 mg/dL; phosphorus = 7.4 ± 0.2 mg/dL) in the absence of treatment (Fig. 4B and C). CTA018/MT2832 administration caused a further increase in mean serum calcium at 2 (to 12 ± 0.4 mg/dL; $p < 0.05$) and 4 (to 12 ± 0.1 mg/dL; $p < 0.05$) weeks only at the highest administered dose (6 μg/kg), but had no effect on serum phosphorus levels at any of the tested doses.

4. Discussion

We have described herein two novel vitamin D analogues, named CTA091 and CTA018/MT2832, which possess potent CYP24

inhibitory activity. These analogues specifically target the unique substrate binding pocket of CYP24, unlike azole-based compounds (e.g., ketoconazole) which target the heme group at the catalytic core of the enzyme. CTA091 does not bind the VDR and, consequently, is unable to induce VDR-mediated gene expression. As we have shown, administration of CTA091 does not induce CYP24 mRNA. Nevertheless, by inhibiting CYP24, CTA091 indirectly lowers serum iPTH levels in normal rats probably by raising serum levels of $1\alpha,25(OH)_2D_3$. When co-administered with $1\alpha,25(OH)_2D_3$, CTA091 potentiates the natural hormone's activity by increasing the hormone's concentration in the blood. Unlike CTA091, CTA018/MT2832 readily binds the VDR and induces VDR-mediated gene expression. As we have shown, administration of CTA018/MT2832 induces CYP24 mRNA. When administered to uremic rats, CTA018/MT2832 effectively suppresses elevated plasma iPTH at doses which have no effect on serum calcium or phosphorus. Therefore, this analog has potential utility in treating SHPT in CKD. A Phase II clinical trial with CTA018/MT2832 is currently in progress in patients with end-stage renal disease and SHPT.

The potential utility of CYP24 inhibitors to treat SHPT is supported by studies in CYP24 knock-out mice which demonstrated a prolonged half-life of systemically administered $1\alpha,25(OH)_2D_3$ and increased responsiveness to administered vitamin D [5,6]. These findings suggest that an inhibitor of CYP24 could effectively treat SHPT in CKD patients having residual renal CYP27B1 activity by potentiating the activity of any hormone still produced. Moreover, CKD patients lacking significant residual renal CYP27B1 might be more effectively treated with an analogue which had both VDR agonist and CYP24 inhibition activities.

A pure CYP24 inhibitor, like CTA091, may be therapeutically useful in combination with vitamin D supplements (e.g., cholecalciferol or ergocalciferol), $25(OH)D_3$ or vitamin D hormone replacement therapies (e.g., doxercalciferol or paricalcitol). By combining CTA091 with $25(OH)D_3$, for example, it might be possible to amplify the renal production of $1\alpha,25(OH)_2D_3$ or in target tissues by reducing the ratio of CYP24 to CYP27B1 activity. This therapeutic approach would be potentially useful in dialysis patients who are essentially "anephric" in terms of renal $1\alpha,25(OH)_2D_3$ production but who retain residual CYP27B1 activity in target tissues. By combining CTA091 with paricalcitol, it might be possible to potentiate iPTH reduction by limiting paricalcitol's self-induced catabolism caused by iatrogenic induction of CYP24 expression in parathyroid glands and other tissues.

We have demonstrated that CTA091 can potentiate the activity of $1\alpha,25(OH)_2D_3$ in rats. Thus, combining a CYP24 inhibitor with vitamin D hormone replacement therapy would provide a means of overcoming vitamin D resistance due to CYP24 expression. This approach would be applicable not only in the treatment of SHPT associated with CKD, but also in the treatment of other diseases, such as psoriasis [18], cancer [9–11] or hypophosphatemia [7,8], where aberrant CYP24 expression has been shown to impose treatment barriers.

By combining the properties of CYP24 inhibition and VDR agonism in the same molecule, we have succeeded in producing a compound, CTA018/MT2832, which is a potent VDR agonist not readily affected by the intracellular expression of CYP24. Although CTA018 binds to the VDR with an affinity that is 15-fold lower than that for $1\alpha,25(OH)_2D_3$, this analogue is approximately 10-fold more potent than $1\alpha,25(OH)_2D_3$ in activating VDR-mediated transcription. It is entirely possible that this apparent discrepancy in VDR binding compared to transcriptional activity is related to the lack of influence that might otherwise be exerted by CYP24. During prolonged treatment, iatrogenic induction of CYP24 can cause treatment-acquired resistance to current vitamin D hormone replacement therapies [19–21]. Analogues such as CTA018/MT2832 may permit the effective management of SHPT without the need for dose escalation caused by CYP24 resistance.

Our findings suggest that it will be important to carefully consider the tight link between vitamin D signaling and catabolism as we design newer, more effective vitamin D analogues for the treatment of SHPT associated with CKD, for cancer and psoriasis, as well as for other diseases where CYP24 may play an etiological role. By targeting CYP24 alone or combining CYP24 inhibition with VDR agonism, we are optimistic that new analogues can meet the spectrum of needs of CKD patients for vitamin D hormone replacement therapy.

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