Journal of Steroid Biochemistry & Molecular Biology 127 (2011) 269–275

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Substitution at carbon 2 of 19-nor-1α,25-dihydroxyvitamin D_3 with 3-hydroxypropyl group generates an analogue with enhanced chemotherapeutic potency in PC-3 prostate cancer cells

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ARTICLE INFO

Article history:
Received 1 May 2011
Received in revised form 22 August 2011
Accepted 24 August 2011

Keywords:
Vitamin D analogue
Proliferation
Invasion
PC-3
Prostate cancer

ABSTRACT

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3(1α,25$(OH)_2D_3$), has anti-proliferative and anti-invasive activities in prostate cancer cells. Because of 1α,25$(OH)_2D_3$ therapeutic potential in treating cancers, numerous analogues have been synthesized with an attempt to increase anti-proliferative and/or decrease calcemic properties. Among these analogues, 19-nor-1α,25$(OH)_2D_2$ while being less calcemic has equivalent potency as 1α,25$(OH)_2D_3$ in several *in vitro* and *in vivo* systems. We recently showed that 19-nor-2α-(3-hydroxypropyl)-1α,25$(OH)_2D_3$ (MART-10) was at least 500-fold and 10-fold more active than 1α,25$(OH)_2D_3$ in inhibiting the proliferation of an immortalized normal prostate PZ-HPV-7 cells and the invasion of androgen insensitive PC-3 prostate cancer cells, respectively. In this study, we further investigated the effects of MART-10 and 1α,25$(OH)_2D_3$ on the dose- and time-dependent induction of *CYP24A1* gene expression in PC-3 prostate cancer cells. We found that MART-10 induced *CYP24A1* gene expression at a lower concentration with a longer duration compared to 1α,25$(OH)_2D_3$, suggesting that MART-10 is less susceptible to CYP24A1 degradation. Molecular docking model of human CYP24A1 and MART-10 indicates that its side chain is far away from the heme ion and is less likely to be hydroxylated by the enzyme. Furthermore, MART-10 was a more potent inhibitor of PC-3 cell proliferation and invasion compared to 1α,25$(OH)_2D_3$. In addition, MART-10 down-regulated matrix metalloproteinase-9 (MMP-9) expression which could be one mechanism whereby MART-10 influences cancer cell invasion. Finally, we observed that subcutaneous administration of MART-10 up-regulated the *CYP24A1* mRNA expression in rat kidneys without affecting their plasma calcium levels. Thus, our findings demonstrate that MART-10 is biologically active *in vivo* and may be an effective vitamin D analogue for clinical trials to treat prostate cancer.

1. Introduction

Prostate cancer ranks as the highest diagnosed cancer among US men with estimated new cases close to 217,700 in 2010, and second only to lung cancer as the cause of male cancer mortality with about 32,000 estimated deaths [1]. Androgen deprivation therapy is recommended for prostate cancer tumors that fail to respond to prostatectomy or radiation. Despite 75% of male patients responding to androgen deprivation therapy, the median remission time is approximately 2 years. Characteristically, the cancer slowly progresses to a hormone refractory phase that continues to grow independent of androgen. Ultimately, the

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doi:10.1016/j.jsbmb.2011.08.010

cancer becomes more invasive and resistant to available therapies. Therefore, current prostate cancer research is actively pursuing strategies to prevent, delay, or ameliorate the development and progression of androgen-independent prostate cancer.

Androgen signaling has been the main target-based therapy to combat prostate cancer. In addition to androgen, prostate cancer cells also respond to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$, calcitriol], the active form of vitamin D_3, through its specific receptor, vitamin D receptor (VDR). VDR belongs to the steroid nuclear receptor superfamily and is a ligand-dependent transcription factor that modulates gene transcription [2,3]. 1α,25$(OH)_2D_3$ plays important roles in regulating cellular proliferation and differentiation [4] besides its classical role in calcium and phosphate homeostasis and bone mineralization [5,6]. In prostate cancer cells, 1α,25$(OH)_2D_3$ increases cell differentiation and apoptosis while proliferation, invasiveness and metastatic potential are decreased in the presence of 1α,25$(OH)_2D_3$ [7]. However, the drawback of using 1α,25$(OH)_2D_3$-based therapy is the risk of causing systemic hypercalcemia and hypercalciuria [8,9]. Thus, less calcemic analogues of 1α,25$(OH)_2D_3$ with potent anti-proliferative activity could be a second line of therapy when androgen deprivation has failed.

Over the last decade or two, investigators have been synthesizing analogues of 1α,25$(OH)_2D_3$ to reduce the calcemic properties of the natural hormone while retaining or even improving the anti-proliferative activity [10,11]. One such analogue that has shown promise is 19-nor-1α,25$(OH)_2D_2$ (Paricalcitol or Zempler) [12]. 19-nor-1α,25$(OH)_2D_2$ has potency similar to 1α,25$(OH)_2D_3$ in inducing vitamin D target genes and suppressing parathyroid hormone secretion in hemodialysis patients with secondary hyperparathyroidism, without inducing hypercalcemia and hyperphosphatemia [13]. In order to develop more potent anti-cancer agents with less calcemic side effect, we have investigated the effects of a series of analogues with A-ring modifications of 19-nor-1α,25$(OH)_2D_3$ at the carbon-2 proximal to the 1-alpha hydroxyl group on prostate cells. We found that one of the 19-nor-1α,25$(OH)_2D_3$ analogues, with a 3-hydroxypropyl group in the alpha C-2 configuration (or MART-10), was approximately 500- to 1000-fold more potent than 1α,25$(OH)_2D_3$ in inhibiting cell proliferation of PZ-HPV-7 prostate cells, a cell line derived from normal prostate epithelial tissue [14]. It also inhibited the proliferation of the androgen-dependent LNCaP prostate cancer cells [15]. Furthermore, MART-10 was shown to be a poor substrate for CYP24A1 in a cell-free reconstituted system, and is likely more resistant to the CYP24A1-mediated degradation pathway [15]. These data illustrate that MART-10 has an enhanced bioavailability and is more potent than 1α,25$(OH)_2D_3$ in normal prostate cells and androgen-dependent prostate cancer cells. In this report, we further demonstrate that MART-10 is also able to impair the proliferation and invasion of the androgen independent prostate cancer cells, PC-3, and more importantly, we show that MART-10 is active *in vivo*. Thus, our findings suggest that MART-10 could be developed and used for the hormone-refractory metastatic prostate cancer therapy.

2. Materials and methods

1α,25$(OH)_2D_3$ was a generous gift from Dr. M. Uskokovic and MART-10 was synthesized by Dr. A. Kittaka [16].

2.1. Cell cultures

The immortalized PC-3 prostate cancer cells (ATCC, Manassas, VA, USA) were maintained in RPMI growth medium (Sigma, St Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) under 5% CO_2 and at 37 °C.

2.2. Cellular proliferation assay

Cell counting was performed with a hemocytometer to assess the anti-proliferative activities of MART-10 and 1α,25$(OH)_2D_3$ as described [15]. Briefly, PC-3 cells were plated at 5000 cells per cm^2 in 35 mm dishes. The cells were then treated with ethanol vehicle or indicated concentrations of 1α,25$(OH)_2D_3$ or MART-10 on days 2, 4, and 6 in a fresh FBS-supplemented medium. On day 8, the medium was removed from cultures. The attached cells were then trypsinized with Trypsin/EDTA at 37 °C and then neutralized with FBS-supplemented growth medium. Each sample of cells was spun down and resuspended in equal volumes of growth medium. Triplicate aliquots were applied to a hemocytometer for cell counting under a light microscope and the counts generated were averaged. The results are expressed as percent of control from three independent determinations. The experiment was repeated at least 2 more times.

2.3. Real-time qPCR analysis

PC-3 cells were cultured and treated as described in the figure legends. Total RNA was extracted using SV Total RNA Isolation kit (Promega, Madison WI, USA). For each sample, cDNA was generated from 2 μg of total RNA using Superscript RNAase H- (Invitrogen, Carlsbad, CA, USA) with random hexamer primers. For each real-time qPCR reaction, 20 ng of single-stranded cDNAs were mixed with 2X SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and an optimal concentration of sequence specific primers. The PCR was performed with an ABI Prism 7500 (Applied Biosystems) as follows: initial denaturation at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 30 s, annealing/extension at 60 °C for 1 min. Human sequence specific primers were as follows: *CYP24A1*, forward 5′-GGC CTG GAT GTC GTA TTT GC-3′ and reverse, 5′-ACA ATC CAA CAA AGA GCC AAA TGC AGT TGA A-3′. To normalize the amount of sample cDNA added to each reaction, Taqman PDAR eukaryotic 18S rRNA (Applied Biosystems), was used as the endogenous control. Relative quantitation of gene expression was calculated using the ΔΔCt method (User Bulletin publication #2, Applied Biosystems). Total RNA from rat kidneys was purified using SV Total RNA Isolation System (Promega, Madison, WI, USA) and cDNA was synthetized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Single-stranded cDNAs were mixed with 2X TaqMan® Universal PCR Master Mix (Applied Biosystems) and optimal concentration of rat *Cyp24a1* specific TaqMan®probe (Rn01423150, Applied Biosystems). The PCR was performed with an ABI Prism 7500 (Applied Biosystems) as follows: 2 min at 50 °C, initial denaturation at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 1 min. Relative *CYP24A1* expression of each sample was calculated by comparing the results to a standard curve produced by serial dilutions using a pool of all cDNA samples and normalized by the amount of 18S on each sample.

2.4. Western blot analysis

Whole cells were lysed in cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing the protease inhibitor PMSF. The resulting samples containing 10 μg of protein were separated by SDS-polyacrylamide gel electrophoresis on a 4% stacking gel/10% mini-gel, transferred to nitrocellulose membranes, blocked with 5% nonfat milk and incubated with primary anti-MMP-9 antibody (M-17, Santa Cruz Biotech, CA, USA) at 4 °C overnight, washed and incubated with secondary HRP conjugated antibody (Cell Signaling, Danvers, MA, USA). The protein bands were visualized using enhanced chemiluminescence detector (ECL-plus, Amersham

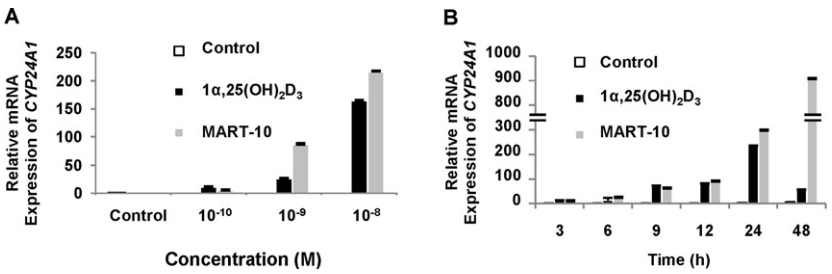

Fig. 1. The dose and time-dependent effect of $1\alpha,25(OH)_2D_3$ and MART-10 on endogenous *CYP24A1* mRNA expression in PC-3 cells. PC-3 cells were treated with ethanol vehicle or the indicated concentrations of $1\alpha,25(OH)_2D_3$ or MART-10 for 24 h (A) or with 10^{-8} M of each substance for the indicated time (h) (B). Then total RNA was prepared and analyzed by relative q-PCR. Data are expressed as the mean ± SD of triplicate determinations. The data were calculated according to the ΔΔCt method and standardized to an endogenous control, S18. Values represent the fold changes in gene expression relative to ethanol vehicle treated control PC-3 cells. The experiment was repeated three times.

Pharmacia Biotech, Piscataway, NJ, USA). Films were then scanned and quantified using Quantity One software (Bio-Rad).

2.5. Animal treatments

Eight weeks-old male Sprague-Dawley rats, weighing ~250 g each, were purchased from Skanbur BK (Sollentuna, Sweden) and the experimental protocol was approved by the Institutional Animal Committee at Karolinska Institute (CFN: N363/08). MART-10 was first dissolved in ethanol and further diluted in propylene glycol to desirable concentrations for subcutaneous (s.c.) injection into animals. A set of control animals were injected s.c. with vehicle alone. At the end of experiments the rats were sacrificed and one kidney was snap frozen in liquid nitrogen. Blood was collected from inferior vena cava into heparinized tubes and after centrifugation supernatants were used for calcium measurement.

2.6. Modeling and docking

Based on the alignment, we constructed a 3D model of human CYP24A1 by mutate monomer using the SYBYL Biopolymer program (Tripos Inc., St. Louis, MO, USA) and the atomic coordinates (Glu19-Arg482) of the crystal structure of rat CYP24A1 (PDB; 3K9V) as a template [17]. The procedure was as follows: First of all, the backbones and side chains were constructed by mutate monomer application so that side chain conformations were retained. Second, hydrogens whose positions were determined by data in SYBYL's internal tables for bond lengths and angles were added. Then, heme was merged into the protein to occupy the same position as the heme of the template protein, rat CYP24A1. Finally, energy minimization of the constructed structure was performed until the energy gradient was lower than 0.1 kcal/(mol Å) using the Tripos force field. The validity of the constructed CYP24A1 model was checked using PROCHECK in the CCP4 program [18]. Docking was performed using Surflex Dock in Sybyl 8.1.1 (Tripos). The human CYP24A1 constructed above was used as the CYP protein structure.

3. Results

3.1. Induction of CYP24A1 gene expression in prostate cancer cells by MART-10

It has been well-established that the *CYP24A1* gene promoter contains vitamin D response elements (VDRE) [19] and is highly inducible by VDR mediated $1\alpha,25(OH)_2D_3$ transactivation [19,20]. Therefore, the induction of *CYP24A1* gene expression has been widely used as a biological marker to monitor the vitamin D mediated gene transactivation [15] and for the comparison of the potency of vitamin D analogues to $1\alpha,25(OH)_2D_3$. Here, we first analyzed the dose responses and time course profiles of $1\alpha,25(OH)_2D_3$ and MART-10 induction of *CYP24A1* gene expression in PC-3 cells using quantitative (q)-PCR (Fig. 1). As shown in Fig. 1A, $1\alpha,25(OH)_2D_3$ and MART-10 at 10^{-10}, 10^{-9}, and 10^{-8} M, caused a dose-dependent increase in the expression of *CYP24A1* by 11.5-, 26.3-, and 164.5-fold, and 5.7-, 87.6-, and 216.8-fold, respectively, as compared to the vehicle control. We observed that MART-10 was significantly more potent than $1\alpha,25(OH)_2D_3$ in inducing *CYP24A1* gene expression at 10^{-9}, and 10^{-8} M ($p < 0.05$).

Interestingly, the time course study (Fig. 1B) demonstrated a bell shape up-regulation of *CYP24A1* gene expression in response to $1\alpha,25(OH)_2D_3$ at 10^{-8} M with a peak response at 24 h, whereas MART-10 at the same concentration continued to increase *CYP24A1* gene expression at 48 h reaching more than 800-fold stimulation. The drastic decrease in the expression at 48 h by $1\alpha,25(OH)_2D_3$ suggests that significant amounts of $1\alpha,25(OH)_2D_3$ were degraded by CYP24A1 resulting from its up-regulation by $1\alpha,25(OH)_2D_3$. The prolonged stimulation by MART-10 suggests that MART-10 may be more resistant to CYP24A1-mediated degradation pathway in agreement with the data obtained from a cell-free reconstituted enzyme system [15]. Overall, these data indicate that MART-10 has enhanced biological activity and remains bioavailable for a longer period of time in these *in vitro CYP24A1*gene expression studies as compared to $1\alpha,25(OH)_2D_3$.

3.2. MART-10 inhibits cellular proliferation and invasion in prostate cancer cells at lower doses compared to$1\alpha,25(OH)_2D_3$

Similar to our previous findings from studies using PZ-HPV-7 and LNCaP cells, MART-10 inhibited PC-3 cell proliferation by 41 ± 7, 42 ± 11, 47 ± 11, and 58 ± 11%, at 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M, respectively, as compared to the controls (Fig. 2).

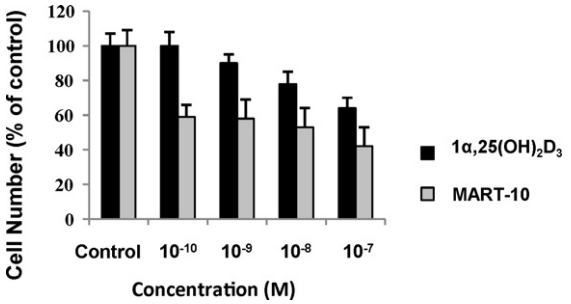

Fig. 2. The dose-dependent effect of $1\alpha,25(OH)_2D_3$ and MART-10 on PC-3 cell proliferation. PC-3 cells were treated with ethanol vehicle or the indicated concentrations of $1\alpha,25(OH)_2D_3$ or MART-10 for 24 h, and then trypsinized, and cell counted with hemocytometer. The results are expressed as percent of control of the means ± SD of 6 determinations. The experiment was repeated three times.

Whereas, 1α,25(OH)$_2$D$_3$ only caused 0 ± 8, 10 ± 5, 22 ± 7, and 36 ± 6% decrease in cell number at the equivalent concentrations. The data clearly show that MART-10 is about 1000-fold more active than 1α,25(OH)$_2$D$_3$ in inhibiting androgen-independent PC-3 cell growth.

3.3. MART-10 regulates matrix metalloproteinase activities

As it has been shown previously in LNCaP cells, MART-10 inhibited PC-3 cell invasion at lower concentrations compared to 1α,25(OH)$_2$D$_3$ [14]. To further investigate the potential mechanisms responsible for the more potent effect of MART-10, we investigated the expression of MMP-9, an enzyme involved in the cell invasion pathway and its expression has been shown to be regulated by 1α,25(OH)$_2$D$_3$ [21]. First, the mRNA level of MMP-9 was measured by q-PCR 24 h after PC-3 cells were treated with 1α,25(OH)$_2$D$_3$ or MART-10 at different concentrations. As shown in Fig. 3, the MMP-9 transcript expression was inhibited by MART-10 by about 40% at 10^{-9} M, whereas no inhibition was observed with 1α,25(OH)$_2$D$_3$ at this concentration. At 10^{-8} M, 1α,25(OH)$_2$D$_3$ exhibited about 35% inhibition which is similar to that induced by MART-10 at 10^{-9} M. Interestingly, no further enhancement of inhibition by MART-10 at 10^{-8} M was observed. On the contrary, the protein expression of MMP-9 as determined by western blot analysis demonstrated a dose-dependent decrease from 10^{-10} M to 10^{-8} M of MART-10 (Fig. 4A and B). A dose-dependent inhibition of MMP-9 protein expression by 1α,25(OH)$_2$D$_3$ was also observed between 10^{-9} M and 10^{-6} M. Therefore, the data suggest that the analogue may exert additional regulation by an unknown post-transcriptional mechanism at the higher concentration. These results indicate that the greater inhibition of MMP-9 activity by MART-10 compared to 1α,25(OH)$_2$D$_3$ at both the mRNA and protein levels may be responsible for the more potent anti-invasion effect observed in the presence of MART-10.

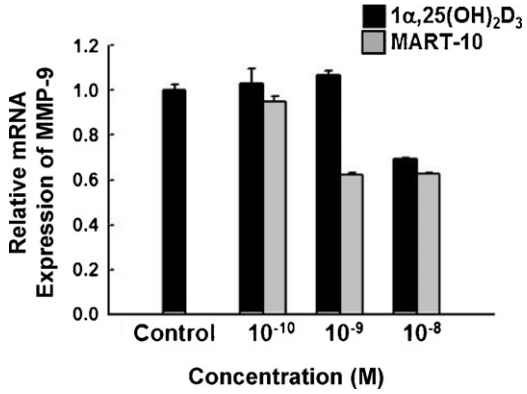

Fig. 3. Relative MMP-9 mRNA expression levels in PC-3 cells treated with MART-10 and 1α,25(OH)$_2$D$_3$ measured by qPCR. PC-3 cells were treated with ethanol vehicle or the indicated concentrations of 1α,25(OH)$_2$D$_3$ or MART-10 for 24 h. Total RNAs were extracted and analyzed for MMP-9 mRNA expression by qPCR. Data are expressed as the mean ± SD of triplicate determinations. The data were calculated according to the ΔΔCt method and standardized to an endogenous control, S18. Values represent the fold changes in gene expression relative to ethanol vehicle treated control PC-3 cells. The experiment was repeated three times.

3.4. MART-10 is biologically active in vivo

19-nor-1α,25(OH)$_2$D$_2$ has been clinically established as a non- or low-calcemic analogue of 1α,25(OH)$_2$D$_3$ [22]. To confirm that MART-10, which is a C-2 modified analogue of 19-nor-1α,25(OH)$_2$D$_3$, is also non or low-calcemic and effective *in vivo* in up-regulating CYP24A1 activity, a marker for MART-10 activity, we evaluated the calcemic activity and *Cyp24a1* mRNA expression in the presence of MART-10 in a rat model. Sprague-Dawley rats were injected subcutaneously with increasing concentrations of MART-10 (0.05, 0.5 and 5 μg/kg), or vehicle as the controls. After 24 h, blood and kidneys were obtained, and plasma calcium and renal

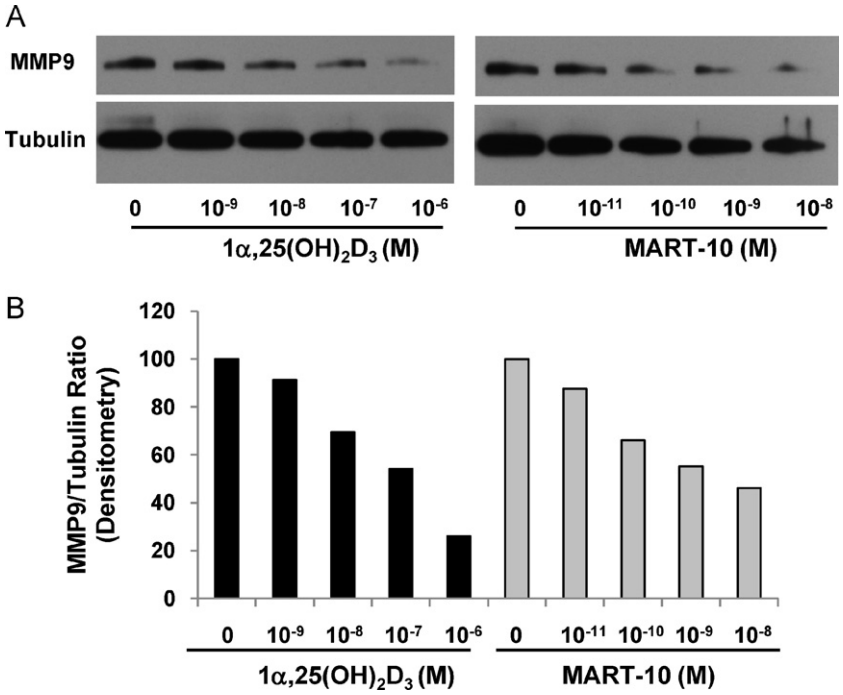

Fig. 4. The dose-dependent effect of 1α,25(OH)$_2$D$_3$ and MART-10 on endogenous MMP-9 protein levels in PC-3 cells. PC-3 cells were treated with ethanol vehicle or the indicated concentrations of 1α,25(OH)$_2$D$_3$ or MART-10 for 24 h, and then whole cell extracts were prepared and analyzed for MMP-9 by western-blot using specific antibodies. Tubulin expression was used as loading control (A). Western-blot results obtained in (A) were quantified using Quantity One software (Bio-Rad) and normalized by the values of the control (B). The experiment was repeated three times.

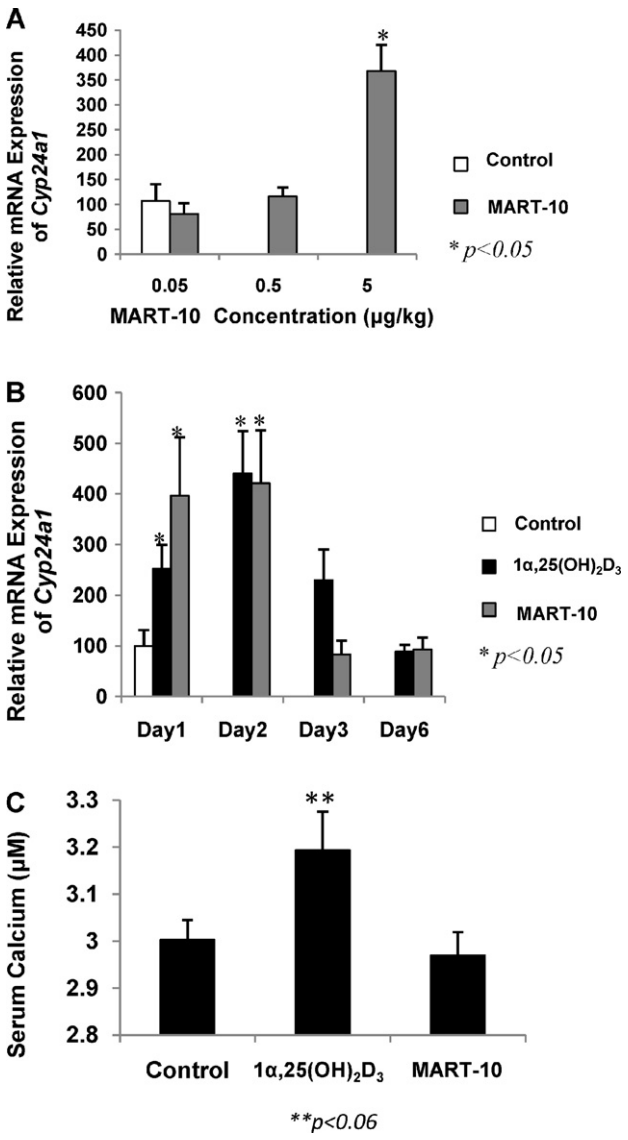

Fig. 5. MART-10 induces *Cyp24a1* expression in Sprague-Dawley rat kidneys. Four Sprague-Dawley rats per group were treated with increasing concentrations of MART-10 or vehicle for 24 h (A) or with 5 μg/kg of MART-10 or 1α,25(OH)$_2$D$_3$ (VD) for the indicated times (days) (B). After the treatment, the rats were sacrificed and total RNA was obtained from the kidneys and analyzed by relative q-PCR. Data are expressed as the mean ± SD of triplicate determinations from the four samples per condition. The data were calculated according to the ΔΔCt method and standardized to an endogenous control, S18 (C). Concentration of calcium in the plasma from the animals studied in (B). Results were presented as mean ± SEM. *Student's *t* test $p < 0.05$ and **Student's *t* test $p < 0.06$ of the sample relative to the control sample.

Cyp24a1 mRNA levels were measured. Fig. 5A shows that MART-10 at a dose of 5 μg/kg body weight induced renal *Cyp24a1 e*xpression 4-fold above basal levels. We next compared the effect of a single concentration (5 μg/kg body) of MART-10 or 1α,25(OH)$_2$D$_3$ on renal *Cyp24a1* expression over a 6-day period (Fig. 5B). At one and two days after the treatment, both 1α,25(OH)$_2$D$_3$ and MART-10 were able to up-regulate *Cyp24a1* expression to a similar extent in a time-dependent manner. However, the stimulatory effect diminished to a level not significantly different from the baseline on days three and six after the treatments (Fig. 5B). Plasma calcium concentration measurement from those animals indicated that MART-10 did not cause an increase in plasma calcium whereas 1α,25(OH)$_2$D$_3$ had a significant elevation in plasma calcium (7%) over the control animals 24 h after the treatment (Fig. 5C). On days 2, 3, and 6, the plasma calcium levels were the same as the controls for both MART-10 and 1α,25(OH)$_2$D$_3$ treated groups (data not shown). Overall our *in vivo* data demonstrate that MART-10 is as effective as 1α,25(OH)$_2$D$_3$ in up-regulating *Cyp24a1*, a sensitive marker of vitamin D biological activity, without raising the blood calcium.

3.5. Docking model of MART-10 to CYP24A1 indicates unlikely hydroxylation at the side-chain by the enzyme

We have previously demonstrated that MART-10 is a much poorer substrate for CYP24A1 than 1α,25(OH)$_2$D$_3$ in a cell-free reconstituted enzyme system [15]. To explain the finding, we conducted docking analysis [23] of MART-10 bound to a computer generated human CPY24A1 based on the crystal structure of rat CYP24A1 reported by Annalora et al. [17]. Two binding modes were obtained by using Surflex Dock software. One docking mode has the side chain of MART-10 positioned over the heme iron atom (data not shown), similar to that of 1α,25(OH)$_2$D$_3$ docked into the rat CYP24A1 substrate binding pocket as reported by Annalora et al. [17]. The other binding mode of MART-10 adopts a different orientation to the first docking mode. In this mode, A-ring is positioned over the heme iron (Fig. 6A), which allows the formation of hydrogen bonds between the 3-hydroxypropyl group of MART-10 and the backbone amide group of Glu329 and carbonyl group of Leu325 as shown in Fig. 6A. Formation of these hydrogen bonds indicates this docking mode is a more favorable one.

As shown in Fig. 6A, Glu329 and Leu325 located in the middle of I helix disrupt α-helix and form a kink. The kink on I helix is widely observed in various CYPs, such as in CYP2A6 as shown in Fig. 6B. This kink motif is responsible for controlling the protonation of oxygen species during oxygen activation. In CYP2A6, conserved threonine (T305; CYP2A6, PDB; 1Z10) on this kink works together with the neighboring conserved glutamic acid residue (E304 in CYP2A6) for oxidation (Fig. 6B) [24]. In addition, conserved threonine residue mediates the hydrogen bonding network as shown in CYP2A6 (Fig. 6B). In the case of rat CYP24A1, the conserved threonine does not contribute to hydrogen bonding network for the kink but instead two water molecules contribute to the hydrogen bonding network (Fig. 6C, PDB; 3K9V) [17].

Intriguingly docked MART-10 shows that oxygen in 3-hydroxypropyl group (Fig. 6A) is over wrapped to one of the water molecules as shown in rat CYP24A1 (Fig. 6C, PDB; 3K9V). The oxygen of the flexible-hydroxypropyl group at C2 of MART-10 forms hydrogen bonds with I-helix residues and these hydrogen bonds stabilize the hydroxypropyl group and I-helix for each other. Consequently the 3-hydroxypropyl group blocks the groove of the I-helix kink and therefore the oxidation step is disrupted.

4. Discussion

In this study we demonstrate that MART-10 inhibits proliferation and markers of invasion of the androgen independent cell line PC-3, indicating that the analogue can inhibit tumorogenic features of prostate cancer cells regardless of their androgen sensitivity. This may suggest that vitamin D analogues are potential candidates for prostate cancer treatments throughout all stages of the disease.

The tumor invasion process is mediated by several groups of proteases: the MMPs, the plasminogen activators and the cathepsins. They are frequently overexpressed in high-grade prostate cancer [25–27]. MMP-9 is one of such proteins and its expression is attenuated by 1α,25(OH)$_2$D$_3$ treatment [21]. In this study we show that MART-10 is able to reduce MMP-9 mRNA (Fig. 3) and protein levels (Fig. 4) at a lower concentration than 1α,25(OH)$_2$D$_3$ in PC-3 cells, suggesting that, the greater inhibition of cell invasion caused by MART-10 may be mediated by the greater inhibition of the MMP-9 expression.

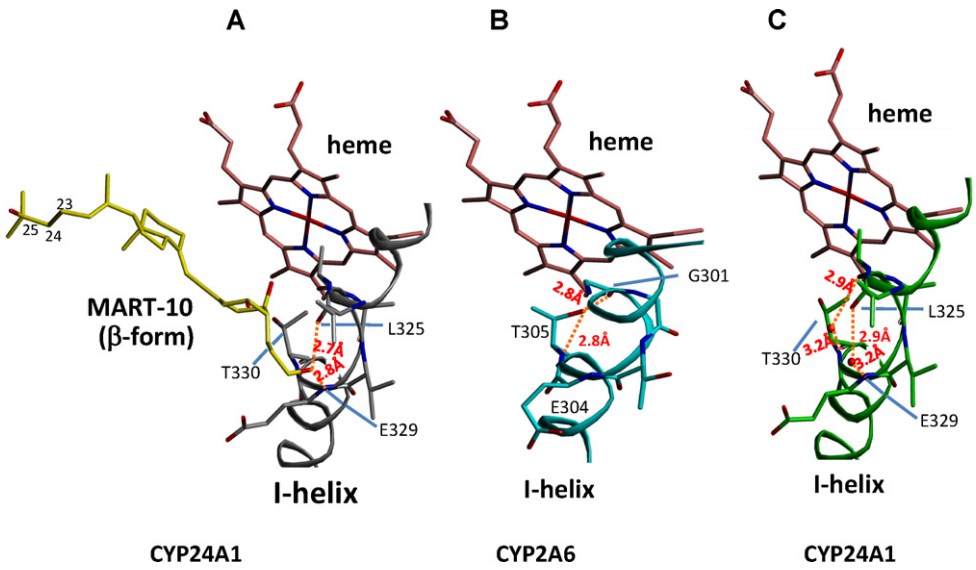

Fig. 6. (A) Docked MART-10 into a modeled human CYP24A1. A-ring of MART-10 is positioned on top of heme group. The oxygen of hydroxypropyl group at the C-2 of the A-ring is located on the groove of I-helix kink. The hydrogen bonds of back bone, L325 and E329, are mediated by hydroxypropyl group. This groove is blocked by hydroxypropyl group. (B) Cyan color shows the I-helix kink in CYP2A6 (PDB; 1Z10). The side chain oxygen of the conserved Thr305 forms hydrogen bonds between the back bone of Gly301 and Thr305. (C) Green color shows I-helix kink in rat CYP24A1 (PDB; 3K9 V). Two water molecules are involved in hydrogen bonding network instead of T330. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Since C-24 hydroxylation by CYP24A1 is responsible for the first step of vitamin D catabolism, one strategy to enhance the potency of vitamin D analogues is to prevent their degradation mediated by this enzyme. Along this line, modification at the side chain containing -ene or -yne groups to prevent 24-hydroxylation has been achieved. For example, 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ was found to be resistant to C-23 and C-24 hydroxylations, and therefore this analogue exhibited 4- to 12-fold higher potency than 1α,25(OH)$_2$D$_3$ [28]. The higher potency exhibited by MART-10 in the current study may result, in part, from its resistant to CYP24A1 mediated degradation (Fig. 1) as this analogue has been shown as a poor substrate for CYP24A1 in a cell-free reconstituted system [15]. Fig. 6A shows the most stable state of MART-10 in the substrate-binding pocket of CYP24A1, in which A-ring of MART-10 is above the heme group and the 3-hydroxypropyl group blocks the groove of the I helix kink, whereas its side chain is far from the heme iron. In contrast, the side chain of 1α,25(OH)$_2$D$_3$ sit in the heme active site of CYP24A1 [17] and is readily subjective to 24-hydroxylation. Thus, based on this docking model, 24-hydroxylation of MART-10 is impossible.

Further enhancement of the MART-10 biological activity may derive from its higher binding affinity to VDR. X-ray crystallographic study indicates an additional hydrogen bonding between the 3-OH of the C-2 substituted 3-hydroxypropyl group and an amino acid lining the VDR binding pocket [29] resulting in higher binding affinity. We have also shown that MART-10 does not bind to vitamin D binding protein (DBP) as tightly as 1α,25(OH)$_2$D$_3$ [15]. This property will allow easier entry of MART-10 into cells or tissues to exert its biological activities. Overall, the higher affinity for VDR, weaker binding to DBP, and resistant to CYP24A1 hydroxylation may contribute to the higher biological activity expressed by MART-10 in the current and previous *in vitro* studies.

Importantly, we demonstrate that MART-10 is biologically active *in vivo* since it is able to induce renal CYP24A1 expression. This finding is the first evidence showing that MART-10 is bioavailable *in vivo*. As opposed to 1α,25(OH)$_2$D$_3$, MART-10 did not elevate the plasma level of calcium in a vitamin D-sufficient rat model [30]. The smaller and slower increase in plasma calcium by 1α,25(OH)$_2$D$_3$ in our study (Fig. 5) compared to that of Brown et al. may be due to a different route of administration (intraperitoneal vs. subcutaneous) [31,32]. Although further investigations are needed to define the full potential and the possible side effects after a long and repeated administration of MART-10, this non-calcemic, long lasting and powerful analogue may be a potential candidate for prostate cancer treatment, especially for patients who do not respond to the conventional therapies or as a complement to those treatments.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science (Nos. 19590016 and 21590022 to A.K.). D. Iglesias-Gato was supported by a postdoctoral fellowship, granted by the Alfornso Martín Escudero Foundation (Spain). Support was also given from VR (2009-3738) and Cancerfonden (Sweden).

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