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Analogs of 1α,25-dihydroxyvitamin D_3 as novel inhibitors of renin biosynthesis

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Abstract

The renin-angiotensin system (RAS) plays a central role in the pathogenesis of hypertension. Recently, we discovered that 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] functions as a negative endocrine regulator of renin biosynthesis, which provides a molecular basis to explore the potential of Vitamin D analogs as renin inhibitors to control the RAS. To search for renin-inhibiting Vitamin D analogs, we screened 20 Vitamin D analog compounds using As4.1-hVDR cell (a juxtaglomerular cell line) culture by Northern blot and luciferase reporter assays. We found that the Gemini compounds, which have two side-chains at carbon-20 position, were particularly active in suppressing renin expression. Eight Gemini compounds were identified that were equally or 10- to 100-times more potent than 1,25$(OH)_2D_3$ in renin inhibition. These Gemini compounds also potently stimulate 25-hydroxyvitamin D 24-hydroxylase expression in As4.1-hVDR cells. Administration of compound RO-27-5646 [1,25-dihydroxy-21-(3-methyl-3-hydroxy-butyl)-19-nor-cholecalciferol] in mice caused a marked reduction in renal renin mRNA expression without invoking severe hypercalcemia as seen in 1,25$(OH)_2D_3$ treatment. These data establish in principle that Vitamin D analogs can indeed inhibit renin expression in vitro and in vivo, and support the notion that low calcemic Vitamin D analogs can potentially be used as therapeutic agents to control the RAS.

Keywords: Vitamin D; Vitamin D analogs; Gemini compounds; Renin; Renin-angiotensin system; Renin inhibitor; As4.1 cells

1. Introduction

Hypertension is a major risk factor for stroke, myocardial infarction, congestive heart failure, progressive atherosclerosis and renal failure, and a leading cause of death in developed countries. One major pathogenic factor contributing to the development of hypertension is the renin-angiotensin system (RAS). The RAS plays a central role in the regulation of blood pressure and electrolyte and volume homeostasis [1], and its inappropriate activation leads to high blood pressure. Given its immense importance in pathogenesis of hypertension, the RAS has been an important drug target for therapeutic intervention of hypertension, with angiotensin-converting enzyme (ACE) inhibitors and angiotensin (Ang) II receptor antagonists being among the most popular anti-hypertensive drugs [2]. In principle, these drugs block the downstream targets of the renin-angiotensin cascade: the ACE and the Ang II receptors. However, besides side effects such as hyperkelemia, cough and rashes, these drugs have some intrinsic problems in their efficacy. For example, Ang II conversion from Ang I can bypass the ACE [3]; since ACE is also involved in the inactivation of bradykinin, ACE inhibitors also affect the kallikrein–kinin system by preventing degradation of bradykinin [4]. On the other hand, since Ang II has multiple receptors with different functions [5], blocking one type of receptor with one antagonist may increase the availability of Ang II to the other type of Ang II receptor, which may potentially enhance the receptor's functions. Therefore, in theory, blocking renin, the first and rate-limiting enzyme of the cascade, might be more effective when the renin level is too high. However, specific renin-inhibiting drugs are not yet

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available. As high-renin hypertension accounts for 10–20% of the patient population with essential hypertension [6], specific inhibitors for renin production are of significant therapeutic values. Such inhibitors, in theory, can be used alone or in combination with ACE inhibitors or Ang II receptor antagonists. Patients with high-renin hypertension generally have higher blood pressure [7] and tend to have a more active sympathetic nervous system [8], thus renin inhibitors may also be used with sympatholytic agents such as the β-blockers in combinational therapy. A great deal of effort had been made in the past to develop renin substrate analogs as renin inhibitors [9–11]; however, these peptide renin inhibitors were not suitable for therapeutic administration to humans.

Recently we discovered that 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the hormonal metabolite of Vitamin D, functions as a negative endocrine regulator of renin biosynthesis. We demonstrated that 1,25$(OH)_2D_3$ specifically suppresses renin gene expression both in vitro and in vivo, and disruption of VDR in mice leads to high blood pressure and cardiac hypertrophy [12–14]. These findings provide a molecular basis to explore the potential of Vitamin D analogs as new renin inhibitors to control the RAS [15]. In theory, analogs with low calcemic effects and potent renin-inhibiting activity are good candidates for renin-targeting agents. Using an in vitro cell culture system, we have screened different Vitamin D analog compounds and identified the Gemini compounds as potent renin inhibitors in both in vitro and in vivo systems.

2. Materials and methods

2.1. Vitamin D and Vitamin D analog compounds

1,25-Dihydroxyvitamin D_3 and Vitamin D analogs listed in Table 1 were provided by BioXell, Inc. (Nutley, NJ) and dissolved in 100% ethanol at 1 mg/ml stock. All stocks were kept at −20 °C in light-insensitive vials.

2.2. Cell culture and treatment

As4.1-hVDR cells, an As4.1 cell clone stably transfected with hVDR cDNA [12], were used in this study. As4.1 cells are a juxtaglomerular cell line derived from kidney tumors of SV40 T antigen transgenic mice [16], which express a high level of endogenous renin. As4.1-hVDR cells were routinely grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS and 350 μg/ml G418. To assess the activity of Vitamin D analog compounds, As4.1-hVDR cells were grown to ∼80% confluence and washed with PBS. The cells were then cultured in DMEM containing 2% charcoal-treated FBS and treated with ethanol (dissolving vehicle for the compounds) or different concentrations of 1,25$(OH)_2D_3$ or analogs (from 10^{-11} to 10^{-8} M) for 24 h. Total cellular RNAs were then extracted at the end of treatment using TRIzol reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

2.3. Northern blot analysis

The levels of steady-state renin mRNA transcript were determined by Northern blot analyses as described previously [12]. Briefly, total RNAs were separated on 1% formaldehyde–agarose gels (15 μg/lane) and transferred onto nylon membranes. The membranes were hybridized with ^{32}P-labeled renin or 25-hydroxyvitamin D 24-hydroxylase (CYP24) cDNA probe. After hybridization the mRNAs were detected by autoradiography and quantified with a PhosphorImager (Molecular Dynamic, Sunnyvale, CA). The same membranes were then striped and rehybridized with ^{32}P-labeled cDNA probe of 36B4, a housekeeping gene [17], for internal loading control. Relative renin mRNA levels were calculated by normalization to 36B4 mRNA levels in each sample.

2.4. Luciferase reporter assays

Renin promoter activity was assessed by luciferase reporter assays as previously described [12] with modifications. Briefly, As4.1-hVDR cells grown in 6-well plates to 70% confluence were co-transfected with pGL3-basic plasmid (control) or pGL-4.1-Luc reporter plasmid (which contains 4.1 kb mouse Ren-1c gene promoter) and pCMV-β-gal plasmid (2.4 μg total plasmid/well) using Lipofectamine (Invitrogen). Twenty-four hours after transfection, one well of transfected cells was split into six wells in 24-well plates, and the cells were grown for 1 day to approximately 80% confluence. Then these cells were treated in duplicates with ethanol, 1,25$(OH)_2D_3$ or analogs at different doses for 36 h in DMEM supplemented with 2% charcoal-treated FBS. At the end of the treatment, the cells were lysed and luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI) and β-galactosidase activity was determined using Gel-Screen Chemiluminescent Reporter System (Tropix, Bedford, MA). Luciferase activity was normalized to β-galactosidase activity in the same cells, and presented as fold induction based on the baseline activity of pGL3-basic vector obtained in the same experiment.

2.5. Animal studies

Three-month old CD-1 male mice (35–40 g) were purchased from Charles River Laboratories (Wilmington, MA). For each experiment, mice were randomly divided into groups of five mice each, and the groups were treated daily with 1,25$(OH)_2D_3$, Vitamin D analog RO-27-5646 (both dissolved in 95% propylene glycol:5% ethanol) or vehicle by intraperitoneal injection (injection volume 50 μl/mouse) at indicated doses. Blood ionized calcium levels were determined every 2–3 days from 50 μl of whole blood using a 634 Ca^{2+}/pH analyzer (Chiron Diagnostics, East Walpole,