The novel vitamin D analog ZK191784 as an intestine-specific vitamin D antagonist

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ABSTRACT Vitamin D [1,25(OH)2D3] plays a crucial role in Ca2+ homeostasis by stimulating Ca2+ (re)absorption and bone turnover. The 1,25(OH)2D3 analog ZK191784 was recently developed to dissociate the therapeutic immunomodulatory activity from the hypercalcemic side effects of 1,25(OH)2D3 and contains a structurally modified side chain characterized by a 22,23-double bond, 24R-hydroxy group, 25-cyclopropyl ring, and 5-butyloxazole unit. We investigated the effect of ZK191784 on Ca2+ homeostasis and the regulation of Ca2+ transport proteins in wild-type (WT) mice and mice lacking the renal epithelial Ca2+ channel TRPV5 (TRPV5+/−). The latter display hypercalciuria, hypercalcemia, vitamin D dependency, increased intestinal expression of the TRPV5 channel, and 1,25(OH)2D3-binding protein calbindin-D28K and intestinal Ca2+ hyperabsorption. ZK191784 normalized the Ca2+ hyperabsorption and the expression of intestinal Ca2+ transport proteins in TRPV5+/− mice. Furthermore, the compound decreased intestinal Ca2+ absorption in WT mice and reduced 1,25(OH)2D3-dependent 45Ca2+ uptake by Caco-2 cells, substantiating a 1,25(OH)2D3-antagonistic action of ZK191784 in the intestine. ZK191784 increased renal TRPV5 and calbindin-D28K expression and decreased urine Ca2+ excretion in WT mice. Both 1,25(OH)2D3 and ZK191784 enhanced transcellular Ca2+ transport in primary cultures of rabbit connecting tubules and cortical collecting ducts, indicating a 1,25(OH)2D3-agonistic effect in kidney. ZK191784 enhanced bone TRPV6 mRNA levels and 1,25(OH)2D3 as well as ZK191784 stimulated secretion of the bone formation marker osteocalcin in rat osteosarcoma cells, albeit to a different extent. In conclusion, ZK191784 is a synthetic 1,25(OH)2D3 ligand displaying a unique tissue-specific profile when administered in vivo. Because ZK191784 acts as an intestine-specific 1,25(OH)2D3 antagonist, this compound will be associated with less hypercalcemic side effects compared with the 1,25(OH)2D3 analogs currently used in clinical practice.—Nijenhuis, T., van der Eerden, B. C. J., Zügel, U., Steinmeyer, A., Weinans, H., Hoenderop, J. G. J., van Leeuwen, J. P. T. M., Bindels, R. J. M. The novel vitamin D analog ZK191784 as an intestine-specific vitamin D antagonist. FASEB J. 20, E1589–E1598 (2006)

Key Words: TRPV5 • TRPV6 • Ca2+ homeostasis • 1,25(OH)2D3.

The main physiological function of the active form of vitamin D [1,25(OH)2D3] is to stimulate intestinal and renal Ca2+ (re)absorption and regulate bone Ca2+ turnover (1, 2). In addition, 1,25(OH)2D3 has potent antiproliferative, immunosuppressive, and immunomodulatory activity (3–5). However, therapeutic administration of 1,25(OH)2D3 frequently has dose-limiting hypercalcemic side effects, increasing the risk of soft-tissue and vascular calcification as well as osteoporosis when administered in a supraphysiological dose (6, 7). Therefore, there has been great effort in identifying new 1,25(OH)2D3 analogs that retain a beneficial therapeutic profile combined with minimal calcemic action. Such analogs would have attractive clinical potential as immunomodulators in hyperproliferative disorders or to treat secondary hyperparathyroidism complicating chronic kidney disease (8).

The 1,25(OH)2D3 analog ZK191784 was developed in an effort to dissociate the immunomodulatory and hypercalcemic actions of 1,25(OH)2D3 (3). This compound contains a structurally modified side chain characterized by a 22,23-double bond, 24R-hydroxy group, 25-cyclopropyl ring, and 5-butyloxazole unit (Fig. 1). ZK191784 competitively binds to the vitamin D receptor (VDR) with a similar affinity as 1,25(OH)2D3 (3). Like 1,25(OH)2D3, ZK191784 inhibited antigen-induced lymphocyte proliferation and cytokine secretion in vitro and exhibited potent immunosuppressive activity in a murine model of contact hypersensitivity. In addition, it exerted a 1,25(OH)2D3-antagonistic effect on the promyelocytic leukemia cell line HL-60. This latter cell model is often used to study the genomic

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doi: 10.1096/fj.06-5515fje

0892-6638/06/0020-1589 © FASEB

E1589
responses of 1,25(OH)2D3 analogs (3, 9), and antagonism of HL-60 differentiation was previously shown with several vitamin D analogs that have 1,25(OH)2D3-antagonistic profiles in vivo (10–13). However, the in vivo effects of ZK191784 regarding Ca2+ homeostasis and regulation of the Ca2+ transport proteins have not been evaluated in detail.

1,25(OH)2D3-stimulated transcellular Ca2+ (re)absorption involves Ca2+ entry across the luminal membrane via the epithelial Ca2+ channels TRPV5 and TRPV6 (1, 14–16). TRPV5 is localized at the luminal membrane of the late distal convoluted tubule (DCT) and connecting tubule (CNT) in kidney. TRPV6 is the homologous epithelial Ca2+ channel localized along the brush-border membrane of the duodenum. After Ca2+ entry across the luminal membrane, Ca2+ bound to Ca2+-binding proteins (calbindins) diffuse to the basolateral membrane of the cell. Ca2+ is finally extruded to the blood compartment by the Na+/Ca2+ exchanger (NCX1) and/or the plasma membrane Ca2+-ATPase (PMCA1b). The stimulatory effect of 1,25(OH)2D3 on the expression of the epithelial Ca2+ channels has been demonstrated and is probably associated with its hypercalcemic side effects (1). Furthermore, recent studies showed the expression of TRPV5 and TRPV6 in bone and demonstrated that TRPV5 is exclusively expressed in osteoclasts where it is involved in osteoclastic bone resorption (17, 18).

TRPV5 knockout (TRPV5−/−) mice display profound renal Ca2+ wasting due to impaired active Ca2+ reabsorption in DCT and CNT (19). Furthermore, elevated serum 1,25(OH)2D3 levels, intestinal Ca2+ hyperabsorption, and reduced bone thickness were demonstrated in these mice. We showed that additional ablation of 25-hydroxyvitamin-D3-1α-hydroxylase (1α-OHase), the renal enzyme responsible for 1,25(OH)2D3 biosynthesis, decreased intestinal TRPV6 expression and Ca2+ absorption in TRPV5−/−/1α-OHase−/− mice (20). Therefore, hypervitaminosis D in these mice appears to represent a compensatory mechanism in an effort to counteract the significant renal Ca2+ leak. Thus, TRPV5−/− mice constitute an ideal animal model to study the effects of compounds with possible 1,25(OH)2D3-antagonistic actions.

The aim of the present study was, therefore, to evaluate the in vivo effect of the novel 1,25(OH)2D3 analog ZK191784 on Ca2+ and bone homeostasis in WT and TRPV5−/− mice. Animals were treated with ZK191784 for 28 days, after which Ca2+ absorption, Ca2+ excretion, and expression of the Ca2+ transport proteins in intestine, kidney, and bone was determined. In addition, bone morphometry was assessed by detailed microcomputed tomographic analysis. Further characterization of the actions of ZK191784 on intestinal, renal, and osteoblast cell lines was performed to reveal the biological profile of this novel 1,25(OH)2D3 analog.

**MATERIALS AND METHODS**

**ZK191784 treatment in TRPV5+/+ (wild-type) and TRPV5−/− mice**

TRPV5−/− mice were generated by targeted ablation of the TRPV5 gene (19). TRPV5+/+ (wild-type) mice and TRPV5−/− littermates were housed in a light and temperature-controlled room with ad libitum access to deionized drinking water and standard pelleted chow (0.25% (wt/v) Na; 1.1% (wt/v) Ca). Ten-week-old TRPV5+/+ and TRPV5−/− mice were treated during 28 days with 50 µg/kg/day ZK191784 (3). Mice were fed for 28 days, after which the animals were housed in metabolic cages enabling ration feeding and collection of 24 h urine samples under mineral oil, preventing evaporation. At the end of the experiment the animals were killed, blood samples were taken, and duodenum, kidney and femur were sampled. The animal ethics board of the Radboud University Nijmegen approved all animal studies.

**Analytical procedures**

Serum and urine Ca2+ concentrations were determined using a colorimetric assay as described previously (21, 22). Mouse serum PTH was measured using an immunoradiometric assay (Inmutopics, San Clemente, CA). Na+ and Li+ concentrations were determined flame-spectrophotometrically (Eppendorf FCM 6343, Hamburg, Germany). Urine pH was measured using an electronic ion analyzer (Hanna Instruments, Szeged, Hungary).

**In vivo 45Ca2+ absorption assay**

Intestinal Ca2+ absorption was assessed by measuring serum 45Ca2+ at early time points after oral gavage. Mice were treated for 28 days with 50 µg/kg/day ZK191784 or vehicle as described above and were fasted 12 h before the experiment. Animals were hemodynamically stable under anesthesia during the assay. The 45CaCl2 was administrated by oral gavage as described previously (19). Blood samples were obtained at indicated time intervals, and serum was analyzed by liquid scintillation counting. The change in the plasma Ca2+ con-
concentration ($\Delta \mu \text{M}$) was calculated from the $^{45}\text{Ca}^{2+}$ content of the serum samples and the specific activity of the administered $\text{Ca}^{2+}$.

**Real-time quantitative polymerase chain reaction analysis**

Total RNA was extracted from duodenum, kidney, and bone using Trizol Total RNA Isolation Reagent (GIBCO, Breda, the Netherlands). Femurs, from which the bone marrow was removed by flushing with PBS, were first homogenized using a Mikro Dismembrator S (Sartorius, Goettingen, Germany). The obtained RNA was subjected to DNase treatment and reverse transcribed (22). Subsequently, the cDNA was used to determine TRPV6 and calbindin-D$_{28K}$ mRNA levels in duodenum, TRPV5 and calbindin-D$_{28K}$ mRNA expression in kidney, and TRPV5 and TRPV6 mRNA in bone by real-time quantitative polymerase chain reaction (PCR) as described previously (22, 23). In addition, mRNA expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was determined as an endogenous control (22, 23). In addition, mRNA expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was determined as an endogenous control, which enabled calculation of specific mRNA expression levels (22, 23). In addition, mRNA expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was determined as an endogenous control, which enabled calculation of specific mRNA expression levels (22, 23).

**Immunohistochemistry**

Staining of kidney sections for TRPV5 and calbindin-D$_{28K}$ was performed on cryosections of periodate-lysine-paraformaldehyde fixed kidney samples as described previously (22, 23). For semiquantitative determination of protein abundance, images were made using a Zeiss fluorescence microscope equipped with a digital camera (Nikon DXM2000i). Images were analyzed with the Image Pro Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD), resulting in quantification of the protein levels as the mean of integrated optical density (IOD).

**Bone analysis**

To evaluate the effects of ZK191784 on bone and the possible correlation between epithelial $\text{Ca}^{2+}$ channel expression and bone homeostasis, femurs from control and ZK191784-treated TRPV5$^{-/-}$ and TRPV5$+/+$ mice were scanned using the SkyScan 1072 microtomograph (SkyScan, Antwerp, Belgium; ref 19). Scans were processed, and a three-dimensional morphometric analysis of the bone was performed, using the 3D-Calculator project free software (http://www.eur.nl/fgg/orthopaedics/Downloads.html). Measured parameters were expressed according to bone histomorphometry nomenclature (24).

$^{45}\text{Ca}^{2+}$ uptake assay in the intestinal Caco-2 cell line

We performed a $^{45}\text{Ca}^{2+}$ uptake assay in the human colon cancer Caco-2 cell line, which displays duodenal characteristics, to determine the effect of ZK191784 in an established intestinal cell model. The $^{45}\text{Ca}^{2+}$ uptake assay was performed as described previously (15). In short, confluent monolayers of Caco-2 cells were incubated for 48 h in normal Dulbecco’s modified Eagle’s medium (DMEM) culture medium (15) or culture medium supplemented with $1 \times 10^{-7} \text{M} \, 1,25(\text{OH})_2\text{D}_3$, $1 \times 10^{-7} \text{M} \, \text{ZK191784}$, or $1 \times 10^{-7} \text{M} \, 1,25(\text{OH})_2\text{D}_3$ together with $1 \times 10^{-7} \text{M} \, \text{ZK191784}$, respectively. Cells were washed with Krebs-Henseleit buffer (KHB) and, subsequently, were preincubated for 8 min in KHB or KHB supplemented with 10 $\mu\text{M}$ of the TRPV6 blocker ruthenium red (Fluka, St. Louis, MO; ref 1). Thereafter, the preincubation buffer was exchanged for $^{45}\text{Ca}^{2+}$ uptake buffer, containing 0.1 mM $\text{CaCl}_2$, 2 mM Na$_2$HPO$_4$, 10 $\mu$M felodipine, 10 $\mu$M verapamil, and 1 $\mu$Ci $^{45}\text{CaCl}_2$. After incubation for 8 min, cells were washed three times with ice-cold stop buffer consisting of KHB supplemented with 0.5 mM CaCl$_2$ and 1.5 mM LaCl$_3$. Cells were lysed in 0.1% (wt/v) SDS, and radioactivity of the lysate was measured using a liquid scintillation counter.

**Transcellular $\text{Ca}^{2+}$ transport in rabbit kidney CNT and CCD primary cell cultures**

Rabbit kidney CNT and CCD tubes were immunodissected from the kidney cortex of New Zealand White rabbits and grown on permeable filters (Costar 0.33 cm$^2$), as described in detail previously (25). Filters containing confluent monolayers of CNT and CCD cells were incubated with normal DMEM culture medium (25) or culture medium supplemented with $1 \times 10^{-7} \text{M} \, 1,25(\text{OH})_2\text{D}_3$, $1 \times 10^{-7} \text{M} \, \text{ZK191784}$, or $1 \times 10^{-7} \text{M} \, 1,25(\text{OH})_2\text{D}_3$ together with $1 \times 10^{-7} \text{M} \, \text{ZK191784}$, respectively. After 48 h of incubation, transepithelial $\text{Ca}^{2+}$ transport was measured during 90 min as described previously.

**Osteocalcin secretion in rat osteosarcoma cells**

Osteocalcin is produced by mature postproliferative osteoblasts at the onset of extracellular matrix (ECM) production. Ligand-induced osteocalcin production by reactive oxygen species (ROS) 17/2.8 cells was used to assess the bone formation potential of ZK191784. ROS 17/2.8 cells were cultured in DMEM culture medium containing 5% fetal bovine serum as described previously (25). Cells were treated with different concentrations of ZK191784 for 48 h, and osteocalcin produced was measured.

**Statistical analysis**

Data are mean ± se. Statistical comparisons were analyzed by one-way ANOVA and Fisher’s multiple comparison. $P$ values <0.05 were considered statistically significant. All analyses were performed using the StatView Statistical Package software (Power PC version 4.51, Berkley, CA) on an Apple iMac computer.

**RESULTS**

**Metabolic studies in ZK191784-treated WT and TRPV5$^{-/-}$ mice**

WT and TRPV5$^{-/-}$ mice were treated for 28 days with 50 $\mu$g/kg/day ZK191784 or vehicle by daily subcutaneous injection. The obtained metabolic data are shown in Fig. 2 and Table 1. Genetic ablation of TRPV5 resulted in a 10-fold increase in $\text{Ca}^{2+}$ excretion compared with WT mice (Fig. 2A). The in vivo $^{45}\text{Ca}^{2+}$ absorption measurements showed a profound enhancement of intestinal $\text{Ca}^{2+}$ absorption in these TRPV5$^{-/-}$ mice.
mice (Fig. 2B). This was accompanied by a minor but significant increase in the serum \( \text{Ca}^{2+} \) concentration (Table 1). ZK191784 treatment in TRPV5\(^{+/+}\) mice normalized the intestinal \( \text{Ca}^{2+} \) hyperabsorption as well as the serum \( \text{Ca}^{2+} \) concentration. In addition, \( \text{Ca}^{2+} \) excretion was decreased by ZK191784 administration in TRPV5\(^{+/+}\) mice but remained significantly elevated compared with WT mice (Fig. 2A). Furthermore, ZK191784 treatment significantly diminished intestinal \( \text{Ca}^{2+} \) absorption and decreased \( \text{Ca}^{2+} \) excretion in WT mice, without altering serum \( \text{Ca}^{2+} \) levels (Fig. 2B; Table 1). Urine volume, \( \text{Na}^+ \) excretion, and \( \text{Li}^+ \) clearance were not affected by ZK191784 in both TRPV5\(^{+/−}\) and TRPV5\(^{++/++}\) mice (Table 1). Furthermore, serum PTH levels did not significantly differ in the treated groups.

**Duodenal mRNA expression of \( \text{Ca}^{2+} \) transport proteins**

To study the effect of ZK191784 on the abundance of \( \text{Ca}^{2+} \) transporters in the intestine, TRPV6 and calbindin-D\(_{9K}\) mRNA expression was determined by real-time quantitative PCR analysis. TRPV5\(^{+/−}\) mice showed profoundly increased TRPV6 (Fig. 3A) and calbindin-D\(_{9K}\) (Fig. 3B) mRNA levels in duodenum compared with WT mice. Administration of ZK191784 to TRPV5\(^{+/−}\) mice significantly reduced the TRPV6 and calbindin-D\(_{9K}\) mRNA abundance, resulting in a complete normalization of the expression of the intestinal \( \text{Ca}^{2+} \) transporters. ZK191784 treatment did not significantly alter TRPV6 and calbindin-D\(_{9K}\) mRNA levels in WT mice.

**Table 1. Serum and urine analysis during ZK191784 treatment in TRPV5\(^{+/+}\) and TRPV5\(^{+/−}\) mice**

<table>
<thead>
<tr>
<th></th>
<th>TRPV5(^{+/+})</th>
<th>ZK191784</th>
<th>Controls</th>
<th>ZK191784</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>( \text{Ca}^{2+} ) (mM)</td>
<td>2.34 ± 0.02</td>
<td>2.36 ± 0.03</td>
<td>2.46 ± 0.03*</td>
<td>2.35 ± 0.03*</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>11 ± 1</td>
<td>16 ± 5</td>
<td>24 ± 6</td>
<td>24 ± 11</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
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<tr>
<td>Diuresis (mL/24 h)</td>
<td>2.7 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>5.4 ± 0.3*</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>( \text{Na}^+ ) excretion (mmol/24 h)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.5 ± 0.1*</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>( \text{Li}^+ ) clearance (µl/min)</td>
<td>14 ± 1</td>
<td>11 ± 2</td>
<td>16 ± 2</td>
<td>13 ± 1</td>
</tr>
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</table>

Controls, mice treated with vehicle only; ZK191784, mice treated for 28 days with 1.25(OH)\(_2\)D\(_3\) analog ZK191784 (50 \( \mu \)g/kg/day). Data are mean ± s.e. *\( P < 0.05 \) vs. TRPV5\(^{+/+}\) controls; #\( P < 0.05 \) vs. TRPV5\(^{+/−}\) controls.
Renal expression of Ca\textsuperscript{2+} transport proteins

To evaluate the effect of ZK191784 on the expression of the Ca\textsuperscript{2+} transport proteins in the kidney, TRPV5 and calbindin-D\textsubscript{28K} mRNA, as well as protein abundance, was determined by real-time quantitative PCR (Fig. 4) and semiquantitative immunohistochemistry, respectively (Fig. 5). ZK191784 significantly increased TRPV5 and calbindin-D\textsubscript{28K} mRNA levels and enhanced protein abundance of these Ca\textsuperscript{2+} transporters in DCT and CNT of WT mice. In contrast, ZK191784 did not significantly alter the reduced calbindin-D\textsubscript{28K} mRNA and protein expression in TRPV5\textsuperscript{-/-} mice.

Expression of TRPV5 and TRPV6 mRNA in bone

Besides duodenum and kidney, bone is an important tissue in Ca\textsuperscript{2+} homeostasis and it was previously shown that the epithelial Ca\textsuperscript{2+} channels TRPV5 and TRPV6 are expressed in this tissue (18). Bone TRPV5 mRNA levels as determined in femur were not affected by ZK191784 in WT mice (Fig. 6A). TRPV5 gene ablation did not alter the TRPV6 mRNA levels in femur (Fig. 6B). However, TRPV6 mRNA expression was significantly enhanced in ZK191784-treated WT and TRPV5\textsuperscript{-/-} mice.

Bone analysis

To evaluate the effects of ZK191784 on bone morphology, femurs were scanned using microcomputed tomography (Fig. 7). Detailed three-dimensional morphometric analysis demonstrated that trabecular thickness (Tb.Th) is reduced in the femoral head of TRPV5\textsuperscript{-/-} mice (Table 2). ZK191784 did not significantly affect the bone morphometric parameters in the femoral head of both mice strains. Analysis of the metaphysis and diaphysis showed that cortical thickness (C.Th) is also significantly reduced in TRPV5\textsuperscript{-/-} mice compared with WT mice but is unaffected by ZK191784 treatment. There were no differences observed in the other trabecular and cortical bone parameters between the treated groups.

\textsuperscript{45}Ca\textsuperscript{2+} uptake assay in the intestinal Caco-2 cell line

To determine the effect of ZK191784 in an isolated intestinal cell model, \textsuperscript{45}Ca\textsuperscript{2+} uptake was determined in the human Caco-2 cell line, which has duodenal characteristics and expresses TRPV6 and calbindin-D\textsubscript{9K} (1, 26). Application of 1 \texttimes 10\textsuperscript{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} for 48 h enhanced the ruthenium red-sensitive \textsuperscript{45}Ca\textsuperscript{2+} uptake, substantiating the presence of 1,25(OH)\textsubscript{2}D\textsubscript{3}-responsive and TRPV6-mediated Ca\textsuperscript{2+} absorption in these polarized epithelial intestinal cells (Fig. 8A). In contrast, incubation with 1 \texttimes 10\textsuperscript{-7} M ZK191784 did not stimulate \textsuperscript{45}Ca\textsuperscript{2+} uptake. Importantly, concomitant application of 1 \texttimes 10\textsuperscript{-7} M ZK191784 with 1 \texttimes 10\textsuperscript{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} significantly inhibited the 1,25(OH)\textsubscript{2}D\textsubscript{3}-dependent \textsuperscript{45}Ca\textsuperscript{2+} uptake by Caco-2 cells.

Transcellular Ca\textsuperscript{2+} transport in rabbit kidney CNT and CCD primary cell cultures

Transcellular Ca\textsuperscript{2+} transport was measured in primary cultures of immunodissected rabbit kidney CNT and CCD cells grown to confluency on permeable filter supports. Application of 1 \texttimes 10\textsuperscript{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} for 48 h enhanced transcellular Ca\textsuperscript{2+} absorption by the confluent monolayers (Fig. 8B). Importantly, 1 \texttimes 10\textsuperscript{-7} M ZK191784 also stimulated Ca\textsuperscript{2+} transport. Addition of 1 \texttimes 10\textsuperscript{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} in the presence of 1 \texttimes 10\textsuperscript{-7} M ZK191784 did not result in a further enhancement of trandepithelial Ca\textsuperscript{2+} transport.

Osteocalcin secretion in rat osteosarcoma cells

Ligand-induced osteocalcin production by ROS 17/2.8 cells was used to assess the potential of ZK191784 to induce bone formation. Osteocalcin is produced by mature osteoblasts at the onset of ECM production. Both 1,25(OH)\textsubscript{2}D\textsubscript{3} and ZK191784 induced osteocalcin production in a dose-dependent manner (Fig. 9). The concentration for half maximal increase (EC\textsubscript{50}) was 2.5 \times 10\textsuperscript{-10} M for 1,25(OH)\textsubscript{2}D\textsubscript{3} and 5.3 \times 10\textsuperscript{-8} M for ZK191784. The efficacy of ZK191784 compared with 1,25(OH)\textsubscript{2}D\textsubscript{3} was 41%.

DISCUSSION

The present study demonstrated that ZK191784 acts as an intestinal 1,25(OH)\textsubscript{2}D\textsubscript{3} antagonist by diminishing...
1,25(OH)2D3-stimulated Ca2+ absorption. Studies in TRPV5−/− mice indicated that this action was achieved by directly down-regulating intestinal Ca2+ transport protein expression. In contrast, in vivo and in vitro experiments indicated that ZK191784 exerts partial agonistic actions on Ca2+ handling in kidney. This tissue-specific partial 1,25(OH)2D3 agonism/antagonism reflects a biological profile unlike any other 1,25(OH)2D3 analog tested so far. Because ZK191784 does not stimulate intestinal Ca2+ absorption, this compound will be associated with less hypercalcemic side effects compared with 1,25(OH)2D3 and its analogs currently used in clinical practice.

1,25(OH)2D3 is an important stimulatory hormone of intestinal Ca2+ absorption and is known to enhance the expression of the duodenal Ca2+ transporters (1). TRPV5−/− mice were previously shown to display hypervitaminosis D due to the profound renal Ca2+ wasting caused by abolishment of active Ca2+ transport in DCT and CNT (19). Indeed, these mice demonstrated significantly enhanced duodenal TRPV6 and calbindin-D9K expression and Ca2+ hyperabsorption. Importantly, the 1,25(OH)2D3 analog ZK191784 normalized the increased expression of the intestinal Ca2+ transporters and, thereby, antagonized intestinal Ca2+ hyperabsorption in TRPV5−/− mice. Furthermore, ZK191784 diminished Ca2+ absorption in WT mice. In contrast, previous studies from our laboratory demonstrated that 1,25(OH)2D3 and several of its analogs enhance Ca2+ transporter expression and intestinal Ca2+ absorption in these mice (1, 27, 28). The fact that duodenal Ca2+ transporter expression was not significantly altered in WT mice suggests that ZK191784 might also antagonize nongenomic effects of 1,25(OH)2D3 in the intestine. This finding is in line with previous studies that suggested that when dietary Ca2+ content is sufficient, there is only limited genomic 1,25(OH)2D3-dependent stimulation of active Ca2+ absorption and, therefore, competitive binding of ZK191784 to the nuclear VDR does not significantly affect Ca2+ transporter expression in these mice (28).

Taken together, these in vivo data indicated that ZK191784 specifically inhibits 1,25(OH)2D3-stimulated intestinal Ca2+ absorption. To test this hypothesis in vitro, we used the intestine-derived Caco-2 cell line, which was previously shown to express both TRPV6 and calbindin-D9K (26). These experiments showed that in the absence of 1,25(OH)2D3, ZK191784 does not affect Ca2+ uptake by these cells. However, applied in combination with 1,25(OH)2D3, ZK191784 was able to significantly diminish 1,25(OH)2D3-stimulated Ca2+ uptake. Thus, the present data demonstrated that ZK191784, unlike 1,25(OH)2D3, does not stimulate Ca2+ uptake by the intestine and exerts a unique antagonistic effect on 1,25(OH)2D3-stimulated active Ca2+ absorption. This can explain why ZK191784 possesses reduced hypercalcemic potency, when administered in a dose known to exert immunosuppressive effects in vivo (3).

ZK191784 reduced renal Ca2+ excretion and significantly enhanced the expression levels of TRPV5 and calbindin-D9K in WT mice. These proteins are tightly regulated by 1,25(OH)2D3 and are crucial for renal Ca2+ reabsorption, which is exemplified by the robust hypercalciuria in TRPV5−/− mice (1, 19). Therefore, the concomitantly increased Ca2+ transporter expression and reduced Ca2+ excretion in WT mice suggested that ZK191784 exerts a 1,25(OH)2D3-agonistic action on renal active Ca2+ reabsorption. The stimulatory effect of ZK191784 on transcellular Ca2+ transport in primary cultures of rabbit CNT and CCD substantiated these findings. Interestingly, ZK191784 did not increase
calbindin-D\textsubscript{28K} abundance in TRPV5\textsuperscript{-/-} mice. However, previous studies from our group demonstrated that blockade of TRPV5-mediated Ca\textsuperscript{2+} influx in DCT and CNT cells down-regulates calbindin-D\textsubscript{28K} expression (29). This indicated that regulation of the latter protein is highly dependent on the magnitude of the Ca\textsuperscript{2+} influx through TRPV5. This could explain the significantly reduced calbindin-D\textsubscript{28K} expression in TRPV5\textsuperscript{-/-} mice, despite elevated 1,25(OH)\textsubscript{2}D\textsubscript{3} levels (19), as well as the absence of a stimulatory effect of ZK191784 in these mice. Interestingly, ZK191784 still resulted in a Ca\textsuperscript{2+}-sparing action in TRPV5\textsuperscript{+/+} mice that, obviously, cannot be explained by stimulation of active Ca\textsuperscript{2+} reabsorption. The unaffected Li\textsuperscript{+} clearance compared with controls, as an inverse measure of proximal tubular Na\textsuperscript{+} reabsorption to which passive Ca\textsuperscript{2+} reabsorption is functionally coupled, does also not support enhanced proximal tubular Ca\textsuperscript{2+} reabsorption. However, abolishment of the compensatory intestinal Ca\textsuperscript{2+} hyperabsorption and reduced serum Ca\textsuperscript{2+} levels will likely result in a decreased filtered Ca\textsuperscript{2+} load and, therefore, would be in line with the decreased Ca\textsuperscript{2+} excretion. Likewise, the fact that ZK191784 had only a small effect on Ca\textsuperscript{2+} excretion in TRPV5\textsuperscript{-/-} mice demonstrated that the 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated Ca\textsuperscript{2+} hyperabsorption does not contribute largely to the hypercalciuria. Together, these findings underline the presence of a primary renal Ca\textsuperscript{2+} leak in TRPV5\textsuperscript{-/-} mice.

The expression of TRPV5 and TRPV6 in bone was previously demonstrated, but the functional role of these epithelial Ca\textsuperscript{2+} channels in this tissue remained unknown (18). In the present study, we showed that TRPV5\textsuperscript{-/-} mice display unaltered bone TRPV6 expression, suggesting that TRPV6 does not compensate for the absence of TRPV5. This argues against redundancy of epithelial Ca\textsuperscript{2+} channels in bone and indicates that both channels play distinct roles in bone homeostasis. Recently, the exclusive expression of TRPV5 in the ruffled border of osteoclasts in bone was demonstrated (17). Furthermore, cultured osteoclasts from TRPV5\textsuperscript{-/-} mice displayed reduced bone resorptive capacity, suggesting that this channel is involved in osteoclastic bone resorption. However, ZK191784 did not alter TRPV5 expression in WT mice but increased bone TRPV6 expression in both mice strains. Ligand-induced osteocalcin production by ROS 17/2.8 cells

**Figure 6.** mRNA expression of epithelial Ca\textsuperscript{2+} channels in bone during treatment with ZK191784 in TRPV5\textsuperscript{+/+} and TRPV5\textsuperscript{-/-} mice. Effect of ZK191784 treatment on mRNA expression of the epithelial Ca\textsuperscript{2+} channels TRPV5 (A) and TRPV6 (B) in bone was determined by real-time quantitative PCR analysis as the ratio of HPRT and depicted as percentage of TRPV5\textsuperscript{+/+} controls. Controls, mice treated with vehicle only; ZK191784, mice treated for 28 days with the 1,25(OH)\textsubscript{2}D\textsubscript{3} analog ZK191784 (50 \textmu g/kg/day). Data are mean ± se. *P < 0.05 vs. TRPV5\textsuperscript{+/+} controls; #P < 0.05 vs. TRPV5\textsuperscript{-/-} controls; n = 9 animals per group.

**Figure 7.** Bone morphometry after treatment with ZK191784 in TRPV5\textsuperscript{+/+} and TRPV5\textsuperscript{-/-} mice. Representative cross-sectional X-ray images of the femoral head (a), lesser trochanter (b), and diaphysis (c) in control and ZK191784-treated TRPV5\textsuperscript{+/+} and TRPV5\textsuperscript{-/-} mice (A). Three-dimensional reconstruction of femurs from control and ZK191784-treated TRPV5\textsuperscript{+/+} and TRPV5\textsuperscript{-/-} mice (B). Controls, mice treated with vehicle only; ZK191784, mice treated for 28 days with the 1,25(OH)\textsubscript{2}D\textsubscript{3} analog ZK191784 (50 \textmu g/kg/day).


**TABLE 2. Bone analysis during ZK191784 treatment in TRPV5+/+ and TRPV5−/− mice**

<table>
<thead>
<tr>
<th></th>
<th>Femoral head</th>
<th>Metaphysis</th>
<th>Diaphysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>ZK191784</td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>TRPV5+/+</td>
<td>TRPV5−/−</td>
<td>TRPV5+/+</td>
</tr>
<tr>
<td>Tb.Th (μm)</td>
<td>84.4 ± 2.1</td>
<td>82.0 ± 2.8</td>
<td>76.7 ± 1.1*</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>0.22 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>CD/TV (mm⁻³)</td>
<td>81 ± 11</td>
<td>112 ± 28</td>
<td>117 ± 15</td>
</tr>
</tbody>
</table>

|                | Controls     | ZK191784   | Controls  | ZK191784 |
|                | TRPV5+/+     | TRPV5−/−   | TRPV5+/+  | TRPV5−/−  |
| Ct.Th (μm)     | 299 ± 12     | 300 ± 7    | 255 ± 10* | 271 ± 8†  |
| Ct.V (mm³)     | 1.9 ± 0.1    | 1.9 ± 0.1  | 1.7 ± 0.1 | 1.7 ± 0.1† |
| Ec.V (mm³)     | 3.2 ± 0.1    | 3.4 ± 0.1  | 3.5 ± 0.1 | 3.2 ± 0.1 |
| Dp.V (mm³)     | 5.1 ± 0.2    | 5.3 ± 0.2  | 5.2 ± 0.2 | 4.9 ± 0.2 |
| Ct.V/Dp.V      | 37.7 ± 0.5   | 36.3 ± 0.6 | 33.2 ± 0.8 | 34.8 ± 0.5 |

Controls, mice treated with vehicle only; ZK191784, mice treated for 28 days with 1,25(OH)₂D₃ analog ZK191784 (50 μg/kg/day). In femoral head, bone volume (BV), total bone marrow volume including trabeculae (TV), trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and connectivity density (CD/TV) (measure of interconnectivity of trabecular network) were determined. In metaphysis and diaphysis, calculations were performed with regard to cortical thickness (Ct.Th), cortical volume (Ct.V), endocortical volume (Ec.V), total thickness (Tb.Th), and connectivity density (CD/TV) (measure of interconnectivity of trabecular network) were determined. In metaphysis and diaphysis, calculations were performed with regard to cortical thickness (Ct.Th), cortical volume (Ct.V), endocortical volume (Ec.V), total thickness (Tb.Th), and connectivity density (CD/TV) (measure of interconnectivity of trabecular network) were determined. In metaphysis and diaphysis, calculations were performed with regard to cortical thickness (Ct.Th), cortical volume (Ct.V), endocortical volume (Ec.V), and cortical bone volume fraction (Ct.V/Dp.V). Data are mean ± se. *P < 0.05 vs. TRPV5+/+ controls; †P < 0.05 vs. ZK191784-treated TRPV5−/−.

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**Figure 8.** Differential effect of 1,25(OH)₂D₃ and ZK191784 on ⁴⁵Ca²⁺ uptake in the intestinal Caco-2 cell line and on transepithelial Ca²⁺ transport in rabbit kidney CNT/CCD primary cell cultures. ⁴⁵Ca²⁺ uptake was determined in Caco-2 cells incubated for 48 h in normal culture medium (Control), culture medium supplemented with 1 · 10⁻⁷ M 1,25(OH)₂D₃, 1 · 10⁻⁷ M ZK191784 or 1 · 10⁻⁷ M 1,25(OH)₂D₃, together with 1 · 10⁻⁷ M ZK191784, respectively (A). Data are depicted as ruthenium red (RR)-sensitive uptake. Transepithelial Ca²⁺ transport was determined in immunodissected rabbit CNT/CCD cultures incubated for 48 h in normal culture medium (Control), culture medium supplemented with 1 · 10⁻⁷ M 1,25(OH)₂D₃, 1 · 10⁻⁷ M ZK191784 or 1 · 10⁻⁷ M 1,25(OH)₂D₃, together with 1 · 10⁻⁷ M ZK191784 (B). Data are mean ± se. *P < 0.05 vs. untreated cells (Control). †P < 0.05 vs. 1,25(OH)₂D₃-treated cells.

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**Figure 9.** Effect of 1,25(OH)₂D₃ and ZK191784 on osteocalcin production in a rat osteosarcoma cell line. Ligand-induced osteocalcin production was determined in ROS 17/2.8 cells stimulated for 72 h with increasing concentrations of 1,25(OH)₂D₃ (▲) or ZK191784 (■) to obtain a dose-response curve (B).
over in ZK191784-treated mice. On the other hand, we cannot exclude that prolonged ZK191784 administration will affect bone mineral density. In addition, morphometric analysis showed that trabecular and cortical thickness was reduced in TRPV5−/− mice. The exact explanation for this bone phenotype remains elusive but in addition to a primary defect due to TRPV5 ablation could be a consequence of the negative Ca2+ balance or a direct result of the hypervitaminosis D. When administered chronically in supraphysiologically concentrations, 1,25(OH)2D3 was previously shown to reduce cortical bone thickness (30).

In summary, this study demonstrated that ZK191784 acts as an intestine-specific 1,25(OH)2D3 antagonist. Similar tissue-specific effects have been described for e.g., the partial estrogen receptor agonist tamoxifen, which was shown to exert a breast-selective, bone-sparing antagonistic action (31–34). Furthermore, our results in TRPV5−/− mice suggest that ZK191784 may ameliorate the clinical picture in disorders that are characterized by high 1,25(OH)2D3 levels or intestinal Ca2+ hyperabsorption. Taken together, the unique properties of this new 1,25(OH)2D3 analog might be of great benefit in clinical practice, where complete inhibition or stimulation, of the 1,25(OH)2D3 endocrine system is mostly undesirable.

The work was financially supported by the Dutch Kidney Foundation (C10.1881, C03.6017) and the Dutch Organization of Scientific Research (Zon-Mw 016.006.001). The authors thank the staff of the Central Animal Facility, Radboud University Nijmegen, for technical support.

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*Received for publication November 25, 2005
Accepted for publication May 15, 2006.*
The novel vitamin D analog ZK191784 as an intestine-specific vitamin D antagonist

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To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.06-5155fje

SPECIFIC AIMS

The main physiological function of vitamin D₃ [1,25(OH)₂D₃] is to stimulate intestinal and renal Ca²⁺ (re)absorption and regulate bone Ca²⁺ turnover. In addition, 1,25(OH)₂D₃ has potent antiproliferative, immunosuppressive, and immunomodulatory activity. However, therapeutic administration of 1,25(OH)₂D₃ has dose-limiting hypercalcemic side effects. Therefore, there has been great effort in identifying new 1,25(OH)₂D₃ analogs that retain a beneficial therapeutic profile with minimal calcemic action. The 1,25(OH)₂D₃ analog ZK191784 was developed in an effort to dissociate the immunomodulatory and hypercalcemic actions of 1,25(OH)₂D₃. However, the in vivo effects of ZK191784 regarding Ca²⁺ homeostasis have not been evaluated in detail.

1,25(OH)₂D₃-stimulated transcellular Ca²⁺ (re)absorption involves Ca²⁺ entry across the luminal membrane via the epithelial Ca²⁺ channels TRPV5 and TRPV6. TRPV5 is localized at the luminal membrane of the late distal convoluted tubule (DCT) and connecting tuft (CNT) in kidney. TRPV6 is the homologous epithelial Ca²⁺ channel localized along the brush-border membrane of duodenum. TRPV5 knockout (TRPV5⁻/⁻) mice display profound renal Ca²⁺ wasting due to impaired active Ca²⁺ reabsorption in DCT and CNT. Furthermore, these mice show hypervitaminosis D leading to intestinal Ca²⁺ hyperabsorption and displayed reduced bone thickness.

The aim of this study was therefore, to evaluate the effect of ZK191784 on Ca²⁺ absorption, Ca²⁺ excretion and expression of the Ca²⁺ transport proteins in intestine and kidney in wild-type (WT) and TRPV5⁻/⁻ mice. Furthermore, the actions of ZK191784 on intestinal, renal, and osteosarcoma cell lines were characterized to reveal the biological profile of this novel 1,25(OH)₂D₃ analog.

PRINCIPAL FINDINGS

1. Metabolic studies in ZK191784-treated WT and TRPV5⁻/⁻ mice

WT and TRPV5⁻/⁻ mice were treated for 28 days with 50 μg/kg/day ZK191784 or vehicle by daily subcutaneous injection. Genetic ablation of TRPV5 resulted in a 10-fold increase in Ca²⁺ excretion compared with WT mice (Fig. 1A) and enhancement of intestinal Ca²⁺ absorption as determined by in vivo ⁴⁵Ca²⁺ absorption measurements (Fig. 1B), accompanied by a minor increase in the serum Ca²⁺ concentration. ZK191784 treatment in TRPV5⁻/⁻ mice normalized the intestinal Ca²⁺ hyperabsorption as well as the serum Ca²⁺ concentration. In addition, Ca²⁺ excretion was decreased by ZK191784 administration in TRPV5⁻/⁻ mice but remained significantly elevated compared with WT mice (Fig. 1A). Furthermore, ZK191784 treatment significantly diminished intestinal Ca²⁺ absorption and decreased Ca²⁺ excretion in WT mice, (Fig. 1B) without altering serum Ca²⁺ levels.

2. ZK191784 inhibits 1,25(OH)₂D₃-stimulated Ca²⁺ absorption and intestinal Ca²⁺ transporter expression

To study the in vivo effect of ZK191784 on the abundance of Ca²⁺ transporters in the intestine, TRPV6 and calbindin-D₉K mRNA expression was determined by real-time quantitative polymerase chain reaction (PCR) analysis. TRPV5⁻/⁻ mice showed profoundly increased TRPV6 and calbindin-D₉K mRNA levels in duodenum compared with WT mice. ZK191784 significantly reduced the TRPV6 and calbindin-D₉K mRNA abundance in TRPV5⁻/⁻ mice, resulting in a complete normali-
human Caco-2 cell line. Application of ZK191784, mice treated for 28 days with the 1,25(OH)\textsubscript{2}D\textsubscript{3} resulted in increased calbindin-D\textsubscript{28K} mRNA as well as protein abundance assay, measuring serum 45Ca\textsuperscript{2+} uptake. TRPV5 activity was determined in metabolic cages (A). Intestinal Ca\textsuperscript{2+} uptake was significantly increased in ZK191784-treated mice. Effect of ZK191784 on renal Ca\textsuperscript{2+} reabsorption in these polarized epithelial intestinal cells (Fig. 2A). Application of ZK191784 significantly enhanced TRPV5 and calbindin-D\textsubscript{28K} in WT mice. Importantly, concomitant application of 1 \times 10^{-7} M ZK191784 also stimulated Ca\textsuperscript{2+} transport in DCT and CNT of WT mice. Ligand-induced osteocalcin production by reactive oxygen species (ROS) 17/2.8 cells was used to assess the potential of ZK191784 to induce bone formation. Osteocalcin is produced by mature osteoblasts at the onset of extracellular matrix production. To evaluate the in vivo effects of ZK191784 on bone morphology, femurs from control and ZK191784-treated mice were scanned using microcomputed tomography. Detailed three-dimensional morphometric analysis demonstrated that both trabecular and cortical bone TRPV5 mRNA levels were not affected by ZK191784 in WT mice, but TRPV6 mRNA expression was significantly enhanced in ZK191784-treated WT and TRPV5−/− mice. 

3. ZK191784 up-regulates renal Ca\textsuperscript{2+} transport proteins and stimulates transcellular Ca\textsuperscript{2+} reabsorption

To evaluate the effect of ZK191784 on the expression of the Ca\textsuperscript{2+} transport proteins in the kidney, TRPV5 and calbindin-D\textsubscript{28K} mRNA as well as protein abundance were determined by real-time quantitative PCR and semiquantitative immunohistochemistry, respectively. ZK191784 significantly increased TRPV5 and calbindin-D\textsubscript{28K} mRNA levels and enhanced protein abundance of these Ca\textsuperscript{2+} transporters in DCT and CNT of WT mice. Transcellular Ca\textsuperscript{2+} transport was measured in primary cultures of immunodissected rabbit kidney CNT and cortical collecting duct (CCD) cells grown to confluency on permeable filter supports. Application of 1 \times 10^{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} for 48 h enhanced transcellular Ca\textsuperscript{2+} absorption by the confluent monolayers (Fig. 2B). Importantly, 1 \times 10^{-7} M ZK191784 also stimulated Ca\textsuperscript{2+} transport. Addition of 1 \times 10^{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} in the presence of 1 \times 10^{-7} M ZK191784 did not result in a further enhancement of transcellular Ca\textsuperscript{2+} transport.

4. Effects of ZK191784 on bone

Bone TRPV5 mRNA levels were not affected by ZK191784 in WT mice, but TRPV6 mRNA expression was significantly enhanced in ZK191784-treated WT and TRPV5−/− mice. Ligand-induced osteocalcin production by reactive oxygen species (ROS) 17/2.8 cells was used to assess the potential of ZK191784 to induce bone formation. Osteocalcin is produced by mature osteoblasts at the onset of extracellular matrix production. The effects of ZK191784 on bone morphology, femurs from control and ZK191784-treated mice were scanned using microcomputed tomography. Detailed three-dimensional morphometric analysis demonstrated that both trabecular and cortical

![Image](https://example.com/image1.png)

**Figure 1.** Renal Ca\textsuperscript{2+} excretion and intestinal Ca\textsuperscript{2+} absorption during treatment with ZK191784 in TRPV5\textsuperscript{+/−} and TRPV5\textsuperscript{−/−} mice. Effect of ZK191784 on renal Ca\textsuperscript{2+} excretion was determined in metabolic cages (A). Intestinal Ca\textsuperscript{2+} absorption was determined in an in vivo 45Ca\textsuperscript{2+} uptake assay, measuring serum 45Ca\textsuperscript{2+} uptake. TRPV5 activity was determined in metabolic cages (B). Data are mean ± SD. *P < 0.05 vs. TRPV5\textsuperscript{+/−} controls; †P < 0.05 vs. TRPV5\textsuperscript{−/−} controls; n = 9 animals per group.

![Image](https://example.com/image2.png)

**Figure 2.** Differential effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} and ZK191784 on 45Ca\textsuperscript{2+} uptake in the intestinal Caco-2 cell line and on transepithelial Ca\textsuperscript{2+} transport in rabbit kidney CNT/CCD primary cell cultures. 45Ca\textsuperscript{2+} uptake was determined in Caco-2 cells incubated for 48 h in normal culture medium (Control) or culture medium supplemented with 1 \times 10^{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3}, 1 \times 10^{-7} M ZK191784 or 1 \times 10^{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} together with 1 \times 10^{-7} M ZK191784, respectively (A). Data are depicted as ruthenium red (RR)-sensitive uptake. Transcellular Ca\textsuperscript{2+} transport was determined in immunodissected rabbit CNT/CCD cultures incubated for 48 h in normal culture medium (Control), culture medium supplemented with 1 \times 10^{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3}, 1 \times 10^{-7} M ZK191784, or 1 \times 10^{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} together with 1 \times 10^{-7} M ZK191784 (B). Data are mean ± SE. *P < 0.05 vs. untreated cells (Control); †P < 0.05 vs. 1,25(OH)\textsubscript{2}D\textsubscript{3}−treated cells.
thickness are reduced in TRPV5−/− mice. ZK191784 did not significantly affect bone morphometric parameters in both mice strains nor were there differences in the other trabecular and cortical bone parameters between the treated groups.

CONCLUSIONS AND SIGNIFICANCE

The present study demonstrated that ZK191784 acts as an intestinal 1,25(OH)2D3 antagonist by diminishing 1,25(OH)2D3-stimulated Ca2+ absorption. Studies in TRPV5−/− mice indicated that this action was achieved by directly down-regulating intestinal Ca2+ transport protein expression. In contrast, ZK191784 exerted partial agonistic actions on Ca2+ handling in kidney and bone. This tissue-specific partial 1,25(OH)2D3 agonism/antagonism reflects a biological profile unlike any other 1,25(OH)2D3 analog used so far (Fig. 3).

ZK191784 normalized the increased expression of the intestinal Ca2+ transporters and, thereby, antagonized the Ca2+ hyperabsorption in TRPV5−/− mice. Previous studies from our laboratory demonstrated that 1,25(OH)2D3 and several analogs enhance Ca2+ transporter expression and intestinal Ca2+ absorption. Furthermore, ZK191784 diminished Ca2+ absorption in WT mice. These data indicated that ZK191784 specifically inhibits 1,25(OH)2D3-stimulated intestinal Ca2+ absorption. To test this hypothesis in vitro, we used the intestine-derived Caco-2 cell line, which expresses TRPV6 and calbindin-D28K. In the absence of 1,25(OH)2D3, ZK191784 did not affect Ca2+ uptake by these cells. However, applied in combination with 1,25(OH)2D3, ZK191784 was able to significantly diminish 1,25(OH)2D3-stimulated Ca2+ uptake. Thus, unlike 1,25(OH)2D3, ZK191784 does not stimulate Ca2+ uptake by the intestine and exerts a unique antagonistic effect on 1,25(OH)2D3-stimulated active Ca2+ absorption.

Renal Ca2+ transporter expression is tightly regulated by 1,25(OH)2D3. The concomitantly increased renal TRPV5 and calbindin-D28K expression accompanied by reduced Ca2+ excretion in WT mice suggested that ZK191784 exerts a 1,25(OH)2D3-agonistic action on renal active Ca2+ reabsorption. The stimulatory effect of ZK191784 on transcellular Ca2+ transport in primary cultures of rabbit CNT and CCD substantiated the in vivo findings. Interestingly, ZK191784 did not increase calbindin-D28K abundance in TRPV5−/− mice. Previous studies from our group demonstrated that calbindin-D28K expression is highly dependent on the TRPV5-mediated Ca2+ influx in DCT and CNT cells. This could explain the significantly reduced calbindin-D28K expression in TRPV5−/− mice, despite elevated 1,25(OH)2D3 levels, and the absence of a stimulatory effect of ZK191784 in these mice. ZK191784 still resulted in a Ca2+-sparing action in TRPV5−/− mice that, obviously, cannot be explained by stimulation of active Ca2+ reabsorption. However, abolition of Ca2+ hyperabsorption and reduced serum Ca2+ results in a decreased filtered Ca2+ load and, therefore, diminished Ca2+ excretion.

The functional role of TRPV5 and TRPV6 in bone remains largely elusive. Recently, it was demonstrated that TRPV5 is exclusively expressed in the ruffled border of osteoclasts and that cultured osteoclasts from TRPV5−/− mice display reduced bone resorptive capacity. ZK191784 did not alter TRPV5 expression but increased bone TRPV6 expression. Ligand-induced osteocalcin production by ROS 17/2.8 cells was used to assess the bone formation properties of ZK191784. Although rat osteosarcoma cells secreted osteocalcin on treatment with 1,25(OH)2D3 and ZK191784, the efficacy of the latter was rather weak. Bone morphometry did not suggest altered bone turnover in ZK191784-treated mice.

In conclusion, this study demonstrated that ZK191784 is an intestine-specific 1,25(OH)2D3 antagonist, being one of few synthetic 1,25(OH)2D3 ligand displaying tissue-specific effects in vivo. These unique properties might be of benefit in clinical practice where complete inhibition, or stimulation, of the 1,25(OH)2D3 endocrine system is mostly undesirable. Our results indicate this compound will display reduced hypercalcemic potential compared with 1,25(OH)2D3 and its analogs currently used in clinical practice.

![Figure 3. Schematic representation of the differential tissue-specific effects of ZK191784 compared with 1,25(OH)2D3 regarding Ca2+ homeostasis and expression of Ca2+ transporters. ZK191784 inhibits 1,25(OH)2D3-stimulated Ca2+ absorption in vivo and in the intestinal Caco-2 cell line and down-regulates 1,25(OH)2D3-stimulated intestinal Ca2+ transporter expression. ZK191784 and 1,25(OH)2D3 both display stimulatory effects in vivo on Ca2+ reabsorption and renal Ca2+ transporter expression as well as in primary cultures of immunodissected rabbit kidney CNT and CCD cells. Furthermore, ZK191784 and 1,25(OH)2D3 stimulate osteocalcin secretion by ROS17/2.8 cells.](image-url)