Antagonistic Actions in Vivo of (23S)-25-Dehydro-1α-Hydroxyvitamin D₃,26,23-Lactone on Calcium Metabolism Induced by 1α,25-Dihydroxyvitamin D₃

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ABSTRACT

The vitamin D analog, (23S)-25-dehydro-1α-hydroxyvitamin D₃-26,23-lactone (TEI-9647), is an antagonist of the 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) nuclear receptor (VDR)-mediated differentiation of human leukemia (HL-60) cells. To clarify whether TEI-9647 could function as an antagonist of 1α,25(OH)₂D₃ in vivo, we investigated in vitamin D-deficient (-D) rats the effects of single doses of TEI-9647 on several parameters of calcium metabolism. TEI-9647 (50 μg/kg iv) acting alone significantly decreased serum phosphate (P) levels, the administration of either TEI-9647, 50 μg/kg, or 1α,25(OH)₂D₃, 0.25 μg/kg, began to decrease the circulating levels by 4 h, which reached a nadir 24 h after administration.

But, when TEI-9647 and 1α,25(OH)₂D₃ were simultaneously administered to -D rats, the TEI-9647 dose-dependently reversed the inhibition of PTH secretion caused by 1α,25(OH)₂D₃, 0.25 μg/kg, at 8 and 24 h after the treatment. In separate experiments, the daily iv administration of 20 μg/kg of TEI-9647 alone to +D rats for 2 weeks resulted in no significant changes in the prevailing serum Ca²⁺ concentration. But doses of 1–20 μg/kg of TEI-9647 in combination with 0.5 μg/kg of 1α,25(OH)₂D₃, for 2 weeks, dose-dependently and significantly suppressed the serum calcium concentration increase caused by the 1α,25(OH)₂D₃. Collectively, these results show that TEI-9647 acting alone displays in vivo weak agonistic actions, but when administered in combination with 1α,25(OH)₂D₃, a potent antagonist of three genomic-mediated calcium metabolism parameters. We conclude that TEI-9647 can also function as an antagonist of 1α,25(OH)₂D₃ in vivo in the rat. (Endocrinology 142: 59–67, 2001)

VITAMIN D is known to undergo a sequential two-step metabolism, in the liver and kidney, to form 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃] or 24R,25-dihydroxyvitamin D₃ [24R,25(OH)₂D₃], respectively (1). To date, 1α,25(OH)₂D₃ is considered to be the most potent metabolite of vitamin D₃ in humans under physiological conditions (24, 25). This major metabolite functions through VDR-mediated genomic responses (5, 6). Recently, we have synthesized various analogs of 1α,25(OH)₂D₃-26,23-lactone to investigate which structural modifications resulted in the modulation of the VDR function. Several compounds were found to have weak agonistic actions (19), including opening of voltage-gated calcium (9) and chloride channels (20), and activation of mitogen-activated protein kinase (MAP kinase) (21).}

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function(s) of 1α,25(OH)2D3-26,23-lactone is/are responsible for its unique biological actions. Among these compounds, two novel 1α,25(OH)2D3-26,23-lactone analogs (see Fig. 1), (23S)-25-dehydro-1α-hydroxy-vitamin D3-26,23-lactone (TEI-9647) and (23R)-25-dehydro-1α-hydroxy-vitamin D3-26,23-lactone (TEI-9648), have been reported to have much stronger 1α,25(OH)2D3 receptor- (VDR) binding affinities than the natural (23S,25R)-1α,25(OH)2D3-26,23-lactone only to fail to induce human promyelocytic leukemia cell (HL-60 cell) differentiation even at high concentration (10−6 m) (31). Intriguingly, both TEI-9647 and TEI-9648 inhibited differentiation of HL-60 cells induced by 1α,25(OH)2D3. In contrast, neither TEI-9647 nor TEI-9648 blocked the actions of retinoic acid and 12-O-tetradecanoylphorbol-13-acetate (TPA) on HL-60 cell differentiation, suggesting that their inhibitory actions might be 1α,25(OH)2D3/VDR specific (31).

Previous reports indicate there exists a vitamin D response element (VDRE) in the promoter region of both the 25-OH-D3-24-hydroxylase gene (32, 33) and the p21 gene (33, 34). Both TEI-9647 and TEI-9648 show significant vitamin D antagonistic activities on 25-OH-D3-24-hydroxylase gene expression regulated by 1α,25(OH)2D3 in HL-60 cells (35) in human osteosarcoma cells (Saos-2 cells and MG-63 cells) (35), in monkey kidney cells (COS-7 cells) (31), and on p21 gene expression induced by 1α,25(OH)2D3 in HL-60 cells (31). Moreover, we recently demonstrated that TEI-9647 can prevent heterodimer complex formation between the VDR and retinoid X receptor (RXR), and subsequent recruitment by the VDR of coactivator proteins like steroid receptor coactivator-1 (SRC-1) (35). These results clearly show that TEI-9647 may be the first antagonist of VDR/VDRE-mediated genomic actions of 1α,25(OH)2D3.

Although it is clear that TEI-9647 and TEI-9648 inhibit the actions of 1α,25(OH)2D3 in vitro as described previously (31, 35), it remains unclear whether they can also act as antagonists to the actions of 1α,25(OH)2D3 in vivo. The objective of the present investigation was to determine whether TEI-9647 could function as an antagonist of 1α,25(OH)2D3 in vivo in -D rats. Here we report that TEI-9647 can inhibit in vivo three key parameters of calcium metabolism, which are all modulated by 1α,25(OH)2D3, namely, stimulation of intestinal calcium absorption, bone calcium mobilization and inhibition of circulating PTH levels.

Materials and Methods

25-Hydroxyvitamin D3 [25(OH)D3], 24R,25-dihydroxyvitamin D3 [24R,25-(OH)2D3], 1α,25(OH)2D3 and (23S,25R)-1α,25(OH)2D3-26,23-lactone were synthesized in our laboratory (36–38). [26,27-methyl-3H]25(OH)D3 (specific activity, 666 GBq/mmol) and [26,27-methyl-3H]1α,25(OH)2D3 (specific activity, 6.66 TBq/mmol) were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). [6α,7α,24R]-1α,25(OH)2D3 (specific activity, 876.7 GBq/mmol) was chemically synthesized by the tritium-labeled sodium borohydride (specific activity, 2.56 TBq/mmol) reduction of (23S,25R)-1α,25(OH)2D3-26,23-lactone (specific activity, 876.7 GBq/mmol) and Calcium-45 were obtained from Amersham International plc (Little Chalfont, Buckinghamshire, UK). Rat PTH immunoradiometric assay kit was purchased from Immunotex (San Clemente, CA).

Determinations of serum concentrations of vitamin D metabolites

Extraction of vitamin D metabolites in serum. Three-to-five milliliters of serum was diluted with two volumes of water and then 50 μl of ethanol containing 5,000 dpm (50 pg) of [26,27-methyl-3H]25(OH)2D3, 4,800 dpm (50 pg) of [26,27-methyl-3H]24R,25(OH)2D3, 4,810 dpm (5 pg) of [26,27-methyl-3H]1α,25(OH)2D3 and 3,000 dpm (25 pg) of [1-3H]25(OH)2D3. The chloroform phase was pooled and evaporated, and the residue was dried by ethanol azeotrope and chromatographed.

Chromatographic purification of vitamin D metabolites. The chloroform extracts were chromatographed on a 1.2 × 10 cm Sephadex LH-20 column eluted with 160 ml of n-hexane:chloroform:methanol (9:1:1). The 25(OH)D3 fraction (8–17 ml), the 24R,25(OH)2D3 fraction (19–60 ml) and the (23S,25R)-1α,25(OH)2D3-26,23-lactone was added in 50 ml glass tube, and the mixture was extracted with 2 volumes of chloroform:methanol (1:1) for 10 min. The chloroform phase was collected and the aqueous phase was re-extracted with 15 ml of chloroform. The chloroform phase was pooled and evaporated, and the residue was dried by ethanol azeotrope and chromatographed.

Preparation of antiserum IgG for calcitroic acid. Calcitroic acid was conjugated with BSA by a mixed anhydride reaction according to Yamamoto et al. (41). Antibodies to calcitroic acid were produced in four rabbits by repeated intradermal injections. The first immunization was performed with 500 μg of conjugate emulsified in Freund’s complete adjuvant and a booster injection was given in a similar manner at 3-week intervals. Antisera were tested frequently for specific binding to [26,27-methyl-3H]1α,25(OH)2D3. On the 7th day after the last booster (the 4th booster), blood was taken from the carotid artery, and the obtained antisera was lyophilized. The antisera dissolved in 0.1 M phosphate buffer (pH 7.0) was applied on Protein A-Sepharose CL-4B (1.5 g). It was washed with 0.1 M phosphate buffer (pH 7.0). The IgG fraction was eluted with 0.1 M glycine HCl buffer (pH 3.0). The eluent was dialyzed with 0.1 M phosphate buffer (pH 7.0) for 24 h at 4 C. After lyophilization, 40 μg of antiserum IgG was obtained. For immunoassay, it was dissolved in 50% aqueous glycerine and the concentration was adjusted to 2.35 mg protein/10 ml and the resulting solution was stored a −20 C until use.

Assay of 25-OH-D, 24R,25(OH)2D3 and 1α,25(OH)2D3. Competitive protein binding assays for 25(OH)D and 24R,25(OH)2D using vitamin D binding protein from the serum of -D rats and RRA for 1α,25(OH)2D using VDR

Fig. 1. Structures of 1α,25(OH)2D3 and the antagonist analogs TEI-9647 and TEI-9648.
prepared from intestinal mucosa of vitamin D-deficient chicks were carried out as described previously (42, 43).

RIA for (23S,25R)-1α,25(OH)2D3, 26,23-lactone. The RIA for (23S,25R)-1α,25(OH)2D3, 26,23-lactone was performed as follows: [26,27-methyl-3H]1α,25(OH)2D3 (25,000 cpm, 26 pg) and various amounts of standard (23S,25R)-1α,25(OH)2D3, 26,23-lactone or (23S,25R)-1α,25(OH)2D3, 26,23-lactone were dissolved in 20 μl of absolute ethanol in 10 × 75 mm glass tubes, and then added 100 μl of 0.01% Triton X-100 containing phosphate buffer A (50 mm phosphate buffer, pH 7.0 and 0.15 m NaCl) was added. Antiserum IgG (200 μl; 23.5 ng protein) for calcitriol acid in phosphate buffer A was added to each assay tube. The assay tubes were incubated for 24 h at 4 C. The free [26,27-methyl-3H]1α,25(OH)2D3 was separated from the IgG bound [26,27-methyl-3H]1α,25(OH)2D3 with 500 μl of dextran-coated charcoal. After incubation at 4 C for 15 min, each tube was centrifuged at 2,260 × g for 10 min at 4 C, and 500 μl of the supernatant was taken and mixed with 10 ml of 1,4-dioxane-based scintillator. The radioactivity was measured with a Beckman Coulter, Inc. liquid scintillation counter (Model LS6500) using an external standard. In this system, the sensitivity of the assay for (23S,25R)-1α,25(OH)2D3, 26,23-lactone was 2.5 pg/tube and measurement of 2.5 to 300 pg/tube was successfully carried out with good reproducibility.

Assay for intestinal calcium transport and bone calcium mobilization. Male weanling Wistar rats were fed a vitamin D-deficient, low calcium diet (Ca2+, 0.0036%; P, 0.3%; Harlan Teklad Research Diet, Madison, WI) for 7 weeks. At the end of the seventh week, a group of three to five rats (each weighing about 100 g) received an iv injection of either 0.5 μg/kg 1α,25(OH)2D3, or 50 μg/kg TEI-9647 in 0.2 ml of 0.2% Triton X-100 saline solution. The rats were killed at the indicated time after the administration and the intestinal calcium transport and serum calcium concentrations were measured. The intestinal calcium transport assay using everted duodenal sacs was carried out by a standard method (44). The serum Ca2+ concentration was determined by the OCPC (O-cresolphthalein complexone) method (45). Under the conditions of the assay elevations in serum calcium are a reflection of bone calcium mobilization (46).

Determinations of PTH concentrations in rat serum. Serum PTH in rats was measured with immunoradiometric assay kits obtained from Immulite (San Clemente, CA) according to their manual.

Statistical analysis

Data are expressed as mean ± S.E.M. The statistical significance of differences between groups was determined using a one-tailed Student’s t test of the STAT VIEW program (Abacus Concepts Inc., Berkeley, CA). A level of P < 0.05 was considered statistically significant.

Experimental animals

All experimental procedures involving animals and the related protocols were approved by the Committee on Animal Care of the Teijin Institute for Biomedical Research Instruments, Inc. (Tokyo, Japan).

Results

The structures of 1α,25(OH)2D3 and the two lactone antagonists are shown in Fig. 1.

Preparation of vitamin D-deficient rats

Vitamin D-deficient (-D) rats were used to investigate the effects of TEI-9647 on calcium metabolism in vivo. When male weanling Wistar rats (4-week-old) were fed a vitamin D-deficient low calcium diet for 3 to 6 weeks, they developed hypocalcemia, hyperparathyroidism, mild alopecia, and rickets.

Table 1 shows concentrations of calcium and four vitamin D metabolites in the serum of normal (+D) rats, and the –D rats fed a –D, low calcium diet for 7 weeks. The serum calcium concentrations of the –D rats were significantly decreased to about one-half that of the +D rats. Similarly, the serum concentrations of vitamin D metabolites in the –D rats were extremely diminished compared with those of the +D rats; particularly the serum concentration of 1α,25(OH)2D3 of the –D rats was 12-fold lower than the +D rats (5.1 ± 3.6 pg/ml vs. 60.3 ± 4.8 pg/ml). The serum concentration of (23S,25R)-1α,25(OH)2D3, 26,23-lactone in the –D rats was undetectable (<5 pg/ml), but in normal rats was 86.8 ± 12.0 pg/ml. In data not presented, we have determined in normal Wistar rats the serum half-lives of single orally administered doses of TEI-9647 (50 μg/kg) and 1α,25(OH)2D3 (0.5 μg/kg) to be 1.1 h and 8.0 h, respectively. The short half-life of TEI-9647 is consistent with its weak binding to the vitamin D binding protein; TEI-9647 binds only 8.4% as well as 1α,25(OH)2D3 to DBP (31).

Effects of TEI-9647 on parameters calcium metabolism in vitamin D-deficient rats

The biological activities of TEI-9647 were assessed in the –D rats that were fed a low calcium diet. Initially, a single dose of 50 μg/kg TEI-9647 was used to test its intestinal calcium transport activity (Fig. 2) or bone calcium mobilization (Fig. 3) in comparison with that of 0.5 μg/kg 1α,25(OH)2D3. As shown in Fig. 2, TEI-9647 acting as a weak agonist slightly but significantly stimulated intestinal calcium transport only 8 h after the administration. In contrast, 1α,25(OH)2D3 slightly stimulated intestinal calcium transport at 4 h, reaching a maximum 8 h after the administration, and then its activity gradually decreased until 72 h. As shown in Fig. 3, TEI-9647, acting as a weak agonist, slightly but significantly raised serum calcium levels after 8 h, however the serum calcium levels returned to the –D baseline level by 24 h. On the other hand, 1α,25(OH)2D3 acting alone generated a remarkable rise in serum calcium levels, which is in accord with previously published studies (26, 27), demonstrating a potent action of 1α,25(OH)2D3 on bone calcium mobilization.

Table 1. Serum concentrations of vitamin D metabolites in normal (+D) and vitamin D-deficient (-D) rats

<table>
<thead>
<tr>
<th>Animals</th>
<th>Serum calcium</th>
<th>25(OH)D</th>
<th>24R,25(OH)2D</th>
<th>1α,25(OH)2D</th>
<th>1α,25(OH)2D-26,23-lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>+D rats</td>
<td>(mg/100 ml)</td>
<td>(ng/ml)</td>
<td>(ng/ml)</td>
<td>(ng/ml)</td>
<td>(pg/ml)</td>
</tr>
<tr>
<td>n = 6</td>
<td>10.4 ± 0.1</td>
<td>22.0 ± 4.4</td>
<td>4.62 ± 0.44</td>
<td>60.3 ± 4.8</td>
<td>86.8 ± 12.0</td>
</tr>
<tr>
<td>-D rats</td>
<td>5.1 ± 0.1*</td>
<td>2.5 ± 0.4*</td>
<td>0.20 ± 0.1*</td>
<td>5.1 ± 3.6*</td>
<td>&lt;5*</td>
</tr>
</tbody>
</table>

Male weanling Wistar rats (4-week-old) raised under incandescent lights were fed for 7 weeks either a normal vitamin D3-containing diet (Nippon Clea Corp., Tokyo, Japan, CE-2; Ca, 1.0%; P, 1.0%; vitamin D3, 2000 IU/kg; Nippon Clea Corp.) or a vitamin D-deficient diet (Ca2+, 0.0036%; P, 0.3%; Harlan Teklad Research Diet, Madison, WI) for 7 weeks ad libitum. Data are expressed as the mean ± S.E.M. Significantly different from the +D rats: *, P < 0.001.
Figures 4 and 5, respectively, indicate the comparative dose-response relationships between TEI-9647 and 1α,25(OH)2D3 for intestinal calcium transport and bone calcium mobilization in the D rats. The intestinal calcium transport of 1α,25(OH)2D3 and TEI-9647 both reached a maximum 8 h after dosing (Fig. 2). Graded doses of TEI-9647 slightly stimulated intestinal calcium transport. The stimulation of intestinal calcium transport by TEI-9647 had only 1/1400 of the activity of 1α,25(OH)2D3 (Fig. 4). Similarly, increasing doses of TEI-9647 resulted in a dose-response elevation of serum calcium concentrations 8 h after the administration. Bone calcium mobilization in the D rats by TEI-9647 was only 1/377 that of 1α,25(OH)2D3 (Fig. 5). These results demonstrate that TEI-9647 acting alone can slightly stimulate intestinal calcium transport and bone calcium mobilization in the D rats.

Effects of TEI-9647 on parameters of calcium metabolism mediated by 1α,25(OH)2D3

Next we examined the antagonistic effects of TEI-9647 on three parameters of calcium metabolism known to be mediated by 1α,25(OH)2D3 in D rats; these are the elevation of both intestinal Ca2+ absorption and bone Ca2+ mobilization, and repression of PTH secretion. Intriguingly, 2–50
μg/kg TEI-9647 dose-dependently inhibited both intestinal calcium absorption and bone calcium mobilization mediated by 1α,25(OH)2D3 24 h after iv dose of 0.25 μg/kg 1α,25(OH)2D3, but could not inhibit these responses 8 h after the treatment (Table 2).

1α,25(OH)2D3 is known to suppress the secretion of PTH and this action can be studied by measuring changes in the serum concentration of immunoreactive PTH (47, 48). Table 3 indicates the time-course changes in the serum PTH levels in the −D rats iv dosed with TEI-9647 or 1α,25(OH)2D3 alone or in combination. When 50 μg/kg TEI-9647 alone was iv administered to the −D rats, there resulted a rapid and marked decrease in serum PTH levels after 4 h which reached a nadir 24 h after the administration. Thereafter, the serum PTH levels gradually recovered to the control levels by 72 h. The same was true in the case of 0.5 μg/kg 1α,25(OH)2D3 acting alone.

Serum calcium levels were only somewhat increased at 8 h after the administration of 50 μg/kg TEI-9647, but after the administration of 0.5 μg/kg 1α,25(OH)2D3, peaked sharply at 8 h and then gradually decreased after 48–72 h to the control levels. The serum PTH and serum calcium levels caused by 1α,25(OH)2D3 showed reciprocal changes with one another.

Table 4 shows the antagonistic effect of TEI-9647 on changes in serum PTH levels mediated by 1α,25(OH)2D3 in the −D rats. When 0.1 to 0.5 μg/kg 1α,25(OH)2D3, or 2 to 50 μg/kg TEI-9647 were separately iv administered to −D rats, serum PTH levels decreased dose-dependently. In contrast, when TEI-9647 and 1α,25(OH)2D3 were both simultaneously iv administered, 2 μg/kg TEI-9647 almost completely reversed the inhibitory action of PTH secretion caused by 0.25 μg/kg 1α,25(OH)2D3 8 h after the treatment. However, at 24 h, this reversal was dose-dependent, from 2 to 50 μg/kg TEI-9647. Fifty μg/kg of TEI-9647 almost completely reversed the action of 0.25 μg/kg 1α,25(OH)2D3.

### Effects of chronic administration of TEI-9647 on serum calcium

Figure 6 presents results describing the consequences on the serum calcium concentration in the rat of the chronic iv administration over a 2-week interval of varying doses of TEI-9647, alone or in combination, with 1α,25(OH)2D3. Figure 6A indicates that the daily iv administration of single doses of TEI-9647, over the range of 1–20 μg/kg, does not result in a significant change in the normal serum calcium level. Thus, in this setting, TEI-9647 is neither an antagonist, nor even a weak agonist against the physiological serum concentrations of 1α,25(OH)2D3 (see Table 1). In contrast (Fig. 6B), when TEI-9647 and 1α,25(OH)2D3 (0.5 μg/kg) are given simultaneously to rats, TEI-9647 only at the high doses (10–20 μg/kg) is able to antagonize the hypercalcemic effects of the exogenously administered 1α,25(OH)2D3.

### Discussion

Recently, we demonstrated that the lactone analog, TEI-9647, which binds 65-fold more strongly to the VDR than the natural (23,25R)-1α,25(OH)2D3-26,23-lactone (31), is an analog which can display varying proportions of weak agonist and strong antagonist properties to the VDR under in vitro conditions. Thus TEI-9647 in HL-60 cell lines in vitro, even at high concentrations, displayed predominantly only antagonist actions (49, 50). But in other cell lines TEI-9647, while a potent antagonist of 1α,25(OH)2D3/VDR, also displayed weak genomic agonist actions (35, 51). Therefore, we have investigated whether TEI-9647 could function as a vitamin D agonist or antagonist in vivo in −D rats.

We used −D rats to assess three parameters of calcium metabolism of TEI-9647 under both vitamin D-deficient and -replete conditions. When the rats were fed a vitamin D-deficient, low calcium diet for 7 weeks, their serum calcium levels and all vitamin D metabolites concentrations were extremely low compared with those of normal +D rats (Table 1). When TEI-9647 was iv administered to these −D rats, TEI-9647 slightly but significantly stimulated intestinal calcium absorption and bone calcium mobilization after 8 h, with a potency of only 1/1400 and 1/377 that of 1α,25(OH)2D3, respectively (Figs. 4 and 5). On the other hand, TEI-9647 dose-dependently inhibited intestinal calcium absorption and bone calcium mobilization 24 h after an iv dose of 0.25 μg/kg 1α,25(OH)2D3, but could not entirely inhibit them after 8 h (Table 2).

It has previously been reported that the time course of 1α,25(OH)2D3-induced intestinal calcium transport is biphasic with peaks of stimulation early at 4–8 h and late at 24 h, which may be reflective of two mechanistically different processes (52–54). The first response by 1α,25(OH)2D3 may initially act by increasing the permeability of the brush border membranes to calcium and may be independent of any de novo genomic actions (55, 56). The second slower response

### Table 2. Effects of TEI-9647 on intestinal calcium absorption and bone calcium mobilization induced by 1α,25(OH)2D3 in vitamin D-deficient rats

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Time (h)</th>
<th>Intestinal Ca2⁺ Absorption (S/M)</th>
<th>Serum Ca2⁺ (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>1.64 ± 0.05</td>
<td>4.67 ± 0.23</td>
</tr>
<tr>
<td>1α,25(OH)2D3</td>
<td>0.25 μg/kg</td>
<td>2.99 ± 0.12³</td>
<td>5.50 ± 0.17³</td>
</tr>
<tr>
<td>TEI-9647</td>
<td>10 μg/kg</td>
<td>1.63 ± 0.06</td>
<td>4.52 ± 0.18</td>
</tr>
<tr>
<td>50 μg/kg</td>
<td>8</td>
<td>1.88 ± 0.14</td>
<td>4.45 ± 0.05</td>
</tr>
<tr>
<td>1α,25(OH)2D3</td>
<td>0.25 μg/kg</td>
<td>2.29 ± 0.12³</td>
<td>5.10 ± 0.10³</td>
</tr>
<tr>
<td>+ TEI-9647</td>
<td>2 μg/kg</td>
<td>2.96 ± 0.23</td>
<td>5.38 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>10 μg/kg</td>
<td>2.92 ± 0.13</td>
<td>5.43 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>50 μg/kg</td>
<td>3.02 ± 0.18</td>
<td>5.64 ± 0.13</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>1.67 ± 0.10</td>
<td>4.64 ± 0.19</td>
</tr>
<tr>
<td>1α,25(OH)2D3</td>
<td>0.25 μg/kg</td>
<td>2.53 ± 0.19³</td>
<td>5.42 ± 0.12³</td>
</tr>
<tr>
<td>+ TEI-9647</td>
<td>2 μg/kg</td>
<td>2.47 ± 0.07</td>
<td>5.31 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>10 μg/kg</td>
<td>2.27 ± 0.23³</td>
<td>4.97 ± 0.18³</td>
</tr>
<tr>
<td></td>
<td>50 μg/kg</td>
<td>2.06 ± 0.08³</td>
<td>4.72 ± 0.21³</td>
</tr>
</tbody>
</table>

After 8 weeks on the vitamin D-deficient, low calcium diet, rats were divided into groups of three or five animals; each rat received a single iv dose of compound(s) in 0.2% Triton X-100-saline solution. Control rats received only vehicle. Eight or 24 h later, the rats were decapitated and intestinal calcium transport and serum calcium concentration were measured as described in Materials and Methods. Data are expressed as mean ± SEM. Significantly different from controls (8 h or 24 h): a P < 0.001 and b P < 0.01. Significantly different from 1α,25(OH)2D3 (8 h or 24 h): c P < 0.01 and d P < 0.05.
mediated by 1α,25(OH)2D3 is believed to involve protein synthesis including calcium binding protein (CaBP) and may, in fact, also depend on the differentiation and maturation of the absorptive cells as they migrate out along the villus (57, 58). The genomic response aspects of the intestinal calcium transport system to 1α,25(OH)2D3 are totally blocked by cycloheximide and partially inhibited by actinomycin D (59).

In the present report, we demonstrated that TEI-9647 did not inhibit the first phase response at 8 h of intestinal calcium absorption induced by 1α,25(OH)2D3, but inhibited the later (24 h) genomic second response of 1α,25(OH)2D3 (Table 2). These results suggest that TEI-9647 could not inhibit the first phase, possibly nongenomic actions of 1α,25(OH)2D3, but did inhibit the genomic actions of 1α,25(OH)2D3. These results are very similar to our earlier report comparing the actions of TEI-9647 and 1α,25(OH)2D3 in NB4 and HL-60 cells to induce cell differentiation (49). Here in HL-60 cells, TEI-9647 antagonized the genomic effect of 1α,25(OH)2D3 in NB4 and HL-60 cells, thought to be mediated by the nongenomic actions of 1α,25(OH)2D3 (49).

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It is widely accepted that 1α,25(OH)2D3 and serum calcium are the major factors that control PTH secretion (60). Silver et al. demonstrated in vivo in normal rats that 1α,25(OH)2D3 dramatically decreased parathyroid gland preproPTH

After 8 weeks on the vitamin D-deficient, low calcium diet, rats were divided into groups of three or five animals; each rat received a single iv dose of compound(s) in 0.2% Triton X-100-saline solution. Control rats received only vehicle. Eight or 24 h later, the rats were decapitated and serum PTH and serum calcium concentrations were measured as described in Materials and Methods. Data are expressed as mean ± sem. Significantly different from controls: *P < 0.05, **P < 0.001, and ***P < 0.001.
mRNA over 3–48 h with no change in serum calcium, and that 1α,25(OH)2D3 directed inhibited PTH gene transcription (5). In this paper, we showed that TEI-9647, acting alone as an agonist, decreased immunoreactive PTH levels over 4 to 24 h with no change in serum calcium, and the serum was obtained. Then, total serum calcium was determined as described in Experimental procedures. Each point is the mean ± SEM of determinations from five rats. **P < 0.01 (significantly different from the control). *P < 0.05, and ***P < 0.01 (significantly different from the 0.5 μg/kg of 1α,25(OH)2D3 group).

To date, only eight candidate antagonists of 1α,25(OH)2D3 biological actions have been reported. 24-Nor-25-hydroxyvitamin D3 (61, 62), (10S)-19-hydroxy-dihydrovitamin D3 (63, 64), and 25-azavitamin D3 (63, 64) were found to inhibit in vivo both intestinal calcium absorption and bone calcium mobilization induced by vitamin D3 or 1α,25(OH)2D3. 1,4-Dihydroxy-3-deoxy-A-homo-19-nor-9,10-seco-cholesta-5,7,12,23-tetraene (65), (23S,25R)-25-hydroxyvitamin D3-26,23-lactone (66) and (23S,25R)-1α,25(OH)2D3-26,23-lactone (26) were found to have a selective inhibitory action in vivo on 1α,25(OH)2D3-mediated bone calcium mobilization. 6-fluoro-vitamin D3 acts in vivo to weakly inhibit intestinal calcium absorption induced by both vitamin D3 and 1α,25(OH)2D3 (67). For these seven analogs, it is not known whether they act only on genomic responses mediated by 1α,25(OH)2D3 or whether they can also antagonize rapid responses; however, no cell- or in vitro-based mechanism-of-action studies have yet been conducted for these eight analogs. In contrast, 1β,25-dihydroxyvitamin D3 [1β,25(OH)2D3] was found to be a potent antagonist of only the nongenomic actions of 1α,25(OH)2D3, such as transcaltachia, 45Ca2+ uptake in ROS 17/2.8 cells, and NB4 cell differentiation, but is unable to block the genomic actions of 1α,25(OH)2D3 (21, 68, 69). However, because all these vitamin D3 analogs have extremely low binding affinities to the VDR, we do not consider that they act as antagonists to 1α,25(OH)2D3 actions through a direct interaction with the nuclear VDR.

We recently demonstrated that TEI-9647 binds much more strongly to the VDR than the naturally occurring metabolite (23S,25R)-1α,25(OH)2D3-26,23-lactone, but does not induce cell differentiation even at high concentrations (10−6 M) (49). Moreover, the differentiation of HL-60 cells induced by 1α,25(OH)2D3 is inhibited by TEI-9647, but not by the natural lactone (31). In separate studies, TEI-9647 (10−7 M) has been found to be an effective antagonist of both 1α,25(OH)2D3 (10−8 M) mediated induction of 25-OH-D3-24-hydroxylase and p21WAF1/CIP1 in HL-60 cells, and activation of the luciferase reporter assay in COS-7 cells and Saos-2 cells transfected with plasmids containing the VDRE of the human and rat 25-OH-D3-24-hydroxylase gene and cDNA of human VDR (31, 35). Moreover, very recently we clearly demonstrated that TEI-9647 inhibits the heterodimer formation between VDR and RXR, and between VDR and SRC-1 in Saos-2 cells (35). Collectively, these results and also molecular modeling of the VDR with TEI-9647 (70), strongly suggest that our novel (23S,25R)-1α,25(OH)2D3-26,23-lactone analog, TEI-9647, is the first documented antagonist of 1α,25(OH)2D3 VDR/VDRE mediated genomic action (31, 35). Importantly, we have shown in this report that TEI-9647 is also an antagonist in vivo of three 1α,25(OH)2D3 calcium metabolism parameters. At present, we are working on further studies concerning the mode of antagonistic action of the weak agonist/strong antagonist TEI-9647.

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