Antagonistic Action of Novel 1α,25-Dihydroxyvitamin D₃-26,23-lactone Analogons on Differentiation of Human Leukemia Cells (HL-60) Induced by 1α,25-Dihydroxyvitamin D₃*

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We examined the effects of two novel 1α,25-dihydroxyvitamin D₃-26,23-lactone (1α,25-lactone) analogues on human promyelocytic leukemia cell (HL-60) differentiation using the evaluation system of the vitamin D nuclear receptor (VDR)/vitamin D-responsive element (DRE)-mediated genomic action stimulated by 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) and its analogues. We found that the 1α,25-lactone analogues (23S)-25-dehydro-1α-hydroxyvitamin D₃-26,23-lactone (TEI-9647), and (23R)-25-dehydro-1α-hydroxyvitamin D₃-26,23-lactone (TEI-9648) bound much more strongly to the VDR than the natural (23S,25R)-1α,25(OH)₂D₃-26,23-lactone, but did not induce cell differentiation even at high concentrations (10⁻⁷ M). Intriguingly, the differentiation of HL-60 cells induced by 1α,25(OH)₂D₃ was inhibited by either TEI-9647 or TEI-9648 but not by the natural lactone. In contrast, retinoic acid or 12-O-tetradecanoylphorbol-13-acetate-induced HL-60 cell differentiation was not blocked by TEI-9647 or TEI-9648. In separate studies, TEI-9647 (10⁻⁷ M) was found to be an effective antagonist of both 1α,25(OH)₂D₃ (10⁻⁸ M) mediated induction of p21WAF1,CIP1 in HL-60 cells and activation of the luciferase reporter assay in COS-7 cells transfected with cDNA containing the DRE of the rat 25(OH)₂D₃-24-hydroxylase gene and cDNA of the human VDR. Collectively the results strongly suggest that our novel 1α,25-lactone analogues, TEI-9647 and TEI-9648, are specific antagonists of 1α,25(OH)₂D₃ action, specifically VDR/DRE-mediated genomic action. As such, they represent the first examples of antagonists, which act on the nuclear VDR.

It is widely accepted that the fundamental biological activities of the hormonal form of vitamin D₃, 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), are to stimulate intestinal calcium absorption and to increase bone calcium mobilization (1, 2). In recent years, however, many new biological functions different from those mentioned above have been reported (3); these include inhibition of cell proliferation and induction of cell differentiation (4), modulation of immunological responses (5), stimulation of insulin secretion (6, 7), and neurobiological functions (8, 9). 1α,25(OH)₂D₃ is believed to mediate biological responses as a consequence of its interaction with both a nuclear receptor (VDR) to regulate gene transcription (10, 11) and with a putative cell membrane receptor to generate rapid non-genomic effects (12), including the opening of voltage-gated calcium and chloride channels (13), and activation of mitogen-activated protein kinase (14).

To better understand the interactions of the ligand/VDR interacting with a vitamin D-responsive element (DRE) located on the promoter of regulated genes, it would be helpful to identify analogues of 1α,25(OH)₂D₃ that can modulate or antagonize these interactions. However, to date the only known antagonist of 1α,25(OH)₂D₃ is the analogue 19,25(OH)₂D₃, which blocks rapid non-genomic responses but is without effect on the classical nuclear VDR (15). (23S,25R)-1α,25-dihydroxyvitamin D₃-26,23-lactone ((23S,25R)-1α,25(OH)₂D₃-26,23-lactone) was found by Ishizuka et al. (16–19) as a major metabolite of 1α,25(OH)₂D₃ both in vivo and in vitro. They reported that the naturally occurring (23S,25R)-1α,25(OH)₂D₃-26,23-lactone has unique biological features in comparison with 1α,25(OH)₂D₃. First of all, the VDR binding affinity of the naturally occurring 1α,25-lactone is very low (17, 20). Nonetheless it can stimulate collagen synthesis in osteoblasts (21, 22) and inhibit formation of osteoclast-like multinucleated cells from bone marrow mononuclear cells and bone resorption induced by 1α,25(OH)₂D₃ (21, 23, 24). It can also stimulate proteoglycan synthesis and type II collagen synthesis in chondrocytes from rabbit costal growth cartilage (25).

Normally proliferating human promyelocytic leukemia cells (HL-60) show promyelocytic features and no differentiated functions (for example, nitro blue tetrazolium (NBT)-reducing activity, monocyte-specific esterase activity, and cell surface

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The abbreviations used are: 1α,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; 1α,25-lactone, 1α,25-dihydroxyvitamin D₃-26,23-lactone; 25(OH)₂D₃-24-hydroxylase, 25-hydroxyvitamin D₃-24-hydroxylase; 25(OH)₂D₃-26,23-lactone, 25-hydroxyvitamin D₃-26,23-lactone; (23S,25R)-1α,25(OH)₂D₃-26,23-lactone, (23S,25R)-1α,25-dihydroxyvitamin D₃-26,23-lactone; 1α,24R, 25(OH)₂D₃, 1α,24R, 25-hydroxyvitamin D₃; 24,25,26,27-tetranor-1α,23,24-trihydroxyvitamin D₃; 24,25,26-27-tetranor-1α,23,24-dihydroxyvitamin D₃; (23S)-1α(OH)₂D₃-26,23-lactone (TEI-9616), (23S)-1α-hydroxyvitamin D₃-26,23-lactone; (23S)-25-dehydro-1α-hydroxyvitamin D₃-26,23-lactone (TEI-9647), (23S)-25-dehydro-1α-hydroxyvitamin D₃-26,23-lactone; (23R)-25-dehydro-1α-hydroxyvitamin D₃-26,23-lactone (TEI-9648), (23R)-25-dehydro-1α,25-dihydroxyvitamin D₃-26,23-lactone; KH-1060, 20-epi-22-oxa-24a,26a-, 27a-trihomo-1α,25(OH)₂D₃; VDR, vitamin D nuclear receptor; DRE, vitamin D-responsive element; DBP, vitamin D-binding protein; NBT, nitro blue tetrazolium; a-NB, α-naphthylbutyrate; TPA, 12-O-tetradecanoylphorbol-13-acetate; ATRA, all-trans retinoic acid; 9-cis-RA, 9-cis-retinoic acid; PCR, polymerase chain reaction; FACS, fluorescent-activated cell sorter; FBS, fetal bovine serum; PBS, phosphate-buffered saline.
marker expression, used as differentiation markers) are detected. However, their differentiation can be induced in vitro by various compounds including all-trans retinoic acid (ATRA), 9-cis-retinoic acid (9-cis-RA) (into granulocytes), 1α,25(OH)2D3, or 12-O-tetradecanoylphorbol-13-acetate (TPA) (into monocyte/macrophages) (4, 26–28). It is well known that HL-60 cells have the VDR, and its cell differentiation is induced by 1α,25(OH)2D3 through a VDR/DRE-mediated pathway (29); as such this is a useful system to study genomic actions of 1α,25(OH)2D3 and related analogues.

We have recently synthesized various analogues of 1α,25-lactone to investigate which functionality of the 1α,25-lactone structure is responsible for its unique biological functions. In this study we report the discovery of the antagonistic biological activities of two novel 1α,25-lactone analogues ((23S)-25-dehydro-1α(OH)25D3-26,23-lactone) (TEI-9647) and ((23S)-25-dehydro-1α(OH)25D3-26,23-lactone) (TEI-9648). These analogues were found to block both 1α,25(OH)2D3-mediated HL-60 cell differentiation and also activation of the luciferase reporter in COS-7 cells that had been transfected with the cDNA containing the DRE of the rat 25(OH)D3-24-hydroxylase gene and cDNA of the human VDR.

EXPERIMENTAL PROCEDURES

Chemicals—25-Hydroxyvitamin D3 (25(OH)D3), 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3), 1α,25(OH)2D3-26,23-lactone, and its analogues (TEI-9616, TEI-9647, and TEI-9648) were synthesized in our laboratory as described previously (20, 30). The chemical structures of 1α,25(OH)2D3-26,23-lactone and its analogues are shown in Fig. 1. The 20-epi-22-oxa-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D3 (KH-1060) was synthesized in our laboratory. TPA and 9-cis-RA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). VDR cDNA was obtained from the CLONTECH Laboratories, Inc. (Palo Alto, CA). 2 mg of total RNA was reverse-transcribed with 50 units of Superscript II (Gibco BRL, Gaithersburg, MD) in 50 ml containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 20 units of RNase inhibitor (RNasin, Promega Corp., Madison, WI), 2.5 mM oligo(dT) primer. Samples were diluted to 100 ml with buffer containing 2 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 50 mM KCl. 100 pmol of each primer and 2.5 units Taq DNA polymerase (Takara Biomedicals, Shiga, Japan) were added, and samples were covered with mineral oil and then subjected to PCR amplification in a programmed thermal cycler. PCR primer was selected with OLIGO™ (National Bioscience), referring to the mRNA sequence registered in GenBank™. For p21WAF1,CIP1 amplification, the PCR primers were 5‘-GGAGGAGGCCCGTGGAGGATGAC and ACAATGTTG- GAGGAGGAGATGC. PCR cycles were as follows: 1 min at 94°C for denaturation, 1 min at 59°C for annealing, 1 min at 72°C for polymerization, 26 cycles. For β-actin amplification, the PCR primers were 5‘-GATATCGCGGCGTCGTGGCAG and CAGGAGAAGACGT- GGAAGGATGC. PCR cycles were as follows: 1 min at 94°C for denaturation, 1 min at 61°C for annealing, 1 min at 72°C for polymerization, 20 cycles. PCR products were analyzed by 2% agarose gel electrophoresis (about 400-base pair product was obtained in p21WAF1,CIP1 PCR and about 800-base pair product was obtained in β-actin PCR).

Luciferase Reporter Gene Assay—The promoter region of the rat 24-hydroxylase gene (−291/+9), which also contains two DREs (gift from Dr. Y. Ohyama, Hiroshima University, Japan (35)) were cloned from an reverse transcribed cDNA (pBR). The DNA sequences of these plasmids were confirmed by sequencing an ABI 373A DNA sequencer (PE Applied Biosystems, Tokyo, Japan). The luciferase activities of the cell lysates were measured with a luciferase assay kit (Toyko Ink Co., Ltd.) according to the manufacturer's instructions. Transfection measurement by luciferase activities was standardized by the galactosidase activities of the same cells determined by β-galactosidase enzyme assay system (Promega).
These plasmids, together with the hVDR expression vector, pSG5hVDR (a gift from Dr. M. R. Haussler, University of Arizona) were introduced into cells by DEAE-dextran. 16 h after the transfection, 10^{-8} M 1a,25(OH)_{2}D_{3} were adjusted by internal -galactosidase activity.

**RESULTS**

Fig. 1 indicates the structures of the naturally occurring 1α,25-lactone and its three analogues. TEI-9616 is a 25-dehydroxylated version of the naturally occurring (23S,25R)-1α,25(OH)_{2}D_{3} that is a naturally occurring metabolite derived from 1α,25(OH)_{2}D_{3} (55).

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(23S,25R)-1α,25(OH)_{2}D_{3} Nuclear Receptor Actions

Fig. 1. Structures of 1α,25(OH)_{2}D_{3}-26,23-lactone analogues. The (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone is a naturally occurring metabolite derived from 1α,25(OH)_{2}D_{3} (55).

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The receptor binding affinities of 1α,25-lactone and its analogues to VDR prepared from HL-60 cells are shown in Fig. 2 and summarized in Table I. The VDR binding affinities of TEI-9647 and TEI-9648 were 10 and 8%, respectively, as compared with 1α,25(OH)_{2}D_{3}. Their binding affinities to VDR of HL-60 cells were 120–140 times stronger than that of the naturally occurring (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone.

In contrast, the binding affinities of TEI-9616 and the naturally occurring (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone to the VDR of HL-60 cells were about 237 (0.48%) and 1,400 (0.07%) times weaker than that of 1α,25(OH)_{2}D_{3}. Similar results were obtained using chick intestinal VDR.

(23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone bound to the plasma DBP 6.2 times stronger than 1α,25(OH)_{2}D_{3}. However, the DBP binding affinities of TEI-9616, TEI-9647, and TEI-9648 are 2.4, 9.3, and 2.5%, respectively, as compared with 1α,25(OH)_{2}D_{3} (Table I).

Our preliminary data indicated that the natural (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone has very weak HL-60 cell differentiation inducing activity (36). From this data and results of the VDR and DBP affinity studies, we predicted that the 1α,25-lactone analogues might be more potent in HL-60 cell differentiation than the natural (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone. We did find that TEI-9616 is a more potent HL-60 cell differentiation agent than the natural (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone; in contrast, neither TEI-9647 nor TEI-9648 could induce cell differentiation even after treatment at 10^{-6} M (data not presented).

In agreement with other studies (37), concentrations of 10^{-9} to 10^{-7} M 1α,25(OH)_{2}D_{3} dose dependently induced differentiation of HL-60 cells; a concentration of 10^{-6} M of 1α,25(OH)_{2}D_{3} differentiated >50% of the cells into NBT-reducing activity positive cells during a 96-h culture period (data not presented). Fig. 3 presents the morphological and histocytochemical changes in HL-60 cells after treatment with TEI-9647 or TEI-9648 in the absence or presence of 10^{-8} M 1α,25(OH)_{2}D_{3}. Although undifferentiated HL-60 cells showed promyelocytic features, cells differentiated by 1α,25(OH)_{2}D_{3} displayed a monocytic appearance (Fig. 3A). However, TEI-9647 and TEI-9648 did not mediate the appearance of any monocyte-like morphological changes even after treatment at 10^{-6} M for 96 h. Surprisingly, the HL-60 cell morphological changes induced by 10^{-8} M 1α,25(OH)_{2}D_{3} were markedly inhibited in the presence of 10^{-6} M TEI-9647 or TEI-9648 (Fig. 3A). Monocytic differentiation markers, such as NBT-reducing activity and α-NB esterase activity, are known to be up-regulated by 1α,25(OH)_{2}D_{3}.

Therefore, we examined the effect of TEI-9647 and TEI-9648 to mediate the up-regulation of differentiation markers induced by 1α,25(OH)_{2}D_{3}. TEI-9647 or TEI-9648 alone could not induce activation of NBT-reducing activity or α-NB esterase activity. In contrast, they both markedly suppressed the up-regulation induced by 1α,25(OH)_{2}D_{3} (Figs. 3, B and C).

Next we examined separately the inhibitory effects of TEI-9647 and TEI-9648 on 1α,25(OH)_{2}D_{3} action in more detail using NBT-reducing activity as a cell differentiation marker. TEI-9647 dose dependently inhibited the cell differentiation induced by 10^{-8} M 1α,25(OH)_{2}D_{3} (Fig. 4A); it caused 40% suppression at 10^{-9} M and almost complete inhibition was observed at 10^{-7} M. Complete suppression was observed at 10^{-6} M TEI-9647. TEI-9648 showed a similar dose-dependent response curve, but its suppressive effect was consistently weaker than that of TEI-9647 (Fig. 4B). In contrast, neither the naturally occurring (23S,25R)-1α,25(OH)_{2}D_{3}-26,23-lactone nor TEI-9616 displayed any ability to inhibit HL-60 cell differentiation, even at 10^{-6} M (data not presented).

A particularly potent analogue of 1α,25(OH)_{2}D_{3} is KH-1060, which has been shown to have a 5,000–10,000-fold more potent cell differentiating activity than 1α,25(OH)_{2}D_{3} (3, 38). As shown in Fig. 5, both TEI-9647 and TEI-9648 could dose dependently (10^{-8}–10^{-6} M) antagonize the HL-60 cell differentiating actions of KH-1060 (3 × 10^{-11} M).

Fig. 6 shows the consequences of TEI-9647 and TEI-9648 on the changes of cell surface marker expression. In HL-60 cells, 1α,25(OH)_{2}D_{3} simultaneously mediates an increase in CD11b expression and a decrease in CD71 expression. Neither TEI-9647 nor TEI-9648 alone could induce such changes of cell surface marker expression (Fig. 6, left side). In contrast, TEI-9647 and TEI-9648 dose dependently blocked the reciprocal changes of CD11b and CD71 expression associated with HL-60 cell differentiation induced by 1α,25(OH)_{2}D_{3}. TEI-9647 completely blocked the increase in CD11b and the decrease in CD71 expression at 10^{-7} M (Fig. 6). Similar results were observed after treatment with TEI-9648, but its potency seemed to be weaker (data not presented).

ATRA and 9-cis-RA are also known to promote cell differentiation of HL-60 cells but into granulocytes. TPA can also induce HL-60 cell differentiation into macrophage-like cells. We examined whether TEI-9647 and TEI-9648 could inhibit HL-60 cell differentiation induced by these compounds. In data not presented, we found that neither TEI-9647 nor TEI-9648 could cause inhibition even after treatment at 10^{-6} M.

Collectively, Figs. 3–6 document the stereospecific ability of...
TEI-9647 and TEI-9648 to inhibit the ability of both 1α,25(OH)2D3 and KH-1060 to mediate the complex process of HL-60 cell differentiation via the VDR. To assess the potential antagonistic action of these two lactone analogues on specific 1α,25(OH)2D3/VDR-activated genes, two separate assays were conducted.

Fig. 7 presents the results of p21WAF1,CIP1 reverse transcription PCR after examining the effect of TEI-9647 on 1α,25(OH)2D3 regulated gene expression. The gene expression of p21WAF1,CIP1 was clearly up-regulated by 10⁻²⁸ M of 1α,25(OH)2D3; whereas 10⁻²⁷ M of TEI-9647 alone did not induce up-regulation of gene expression. Impressively, TEI-9647 clearly suppressed p21WAF1,CIP1 gene expression induced by 1α,25(OH)2D3.

Fig. 8 reports the antagonistic action of TEI-9647 on 1α,25(OH)2D3/VDR-DRE-mediated expression of the 25(OH)D3-24-hydroxylase gene after plasmid transfection in COS-7 cells as evaluated by a luciferase reporter assay. In the absence of TEI-9647, 1α,25(OH)2D3, 10⁻²⁸ M effected a 600-fold increase in 24-hydroxylase reporter gene expression after 48 h. TEI-9647 acting alone had no discernible effect on the 24-hydroxylase gene expression. Impressively 10⁻²⁷ M TEI-9647 inhibited by 50% the 24-hydroxylase activity induced by 10⁻²⁸ M.

The results presented in Figs. 7 and 8 suggest that TEI-9647 is a specific antagonist for VDR/DRE activation of gene expression. However, an alternative interpretation for these results is that the TEI-9647 enhances the catabolism of 1α,25(OH)2D3 in the cell culture system over 24 h to 48 h. Thus, in light of the results presented in Figs. 7 and 8, it is possible that the apparent inhibition of the 1α,25(OH)2D3 agonist effect could have been due to a reduction in the effective concentration of the secosteroid. However, in light of the results presented in Figs. 7 and 8, it is possible that the apparent inhibition of the 1α,25(OH)2D3 agonist effect could have been due to a reduction in the effective concentration of the secosteroid.
sented in Fig. 9, this seems not to be a valid concern. Fig. 9 evaluates the catabolism of [1\(^{-3}\)H]1\(\alpha\),25(OH)\(_2\)D\(_3\) induced by the 1\(\alpha\),25-lactone analogues in HL-60 cells. When TEI-9647, TEI-9648, TEI-9616, or (23S,25R)-1\(\alpha\),25(OH)\(_2\)D\(_3\)-26,23-lactone were separately added to the [1\(^{-3}\)H]1\(\alpha\),25(OH)\(_2\)D\(_3\), there was a consistent reduction in the rate of catabolism of the

| Assay                        | Relative activity |
|------------------------------|-------------------|
| 1\(\alpha\),25(OH)\(_2\)D\(_3\) | TEI-9616 | TEI-9647 | TEI-9648 | (23S,25R)-1\(\alpha\),25(OH)\(_2\)D\(_3\)-26,23-lactone* |
| VDR binding affinity        | 100 | 0.42 | 10.0 | 8.3 | 0.07 |
| Chick intestine VDR         | 100 | 0.48 | 10.2 | 7.2 | 0.14 |
| DBP binding affinity        | 100 | 2.4  | 9.3  | 2.5 | 620 |
| HL-60 Cell differentiation* | 100 | 1.6 (Not induced) | (Not induced) | 0.5 |

* This is a naturally occurring vitamin D metabolite (18). The structures of the lactone-metabolite and its three analogs are given in Fig. 1.

* The summary is presented from data similar to that shown in Figs. 3 and 4.
[1-3H]1α,25(OH)2D3. These results suggest that the antagonistic actions of TEI-9647 and TEI-9648 occur despite blocking of the metabolism of 1α,25(OH)2D3.

**DISCUSSION**

In the process of characterizing the relative importance of the 26,23-lactone ring present on the naturally occurring metabolite (23S,25R)-1α,25(OH)2D3-26,23-lactone, we chemically synthesized two lactone analogues that had a 25-dehydrated (TEI-9647 and TEI-9648). Although the two 25-dehydro diastereoisomers bound to the VDR 115–140-fold better than the natural (23S,25R)-1α,25(OH)2D3 for 96 h, and cell surface marker expression was examined by the FACS analysis. A, changes of CD11b expression; B, changes of CD71 expression. 15,000 cells were analyzed in each analysis.

**FIG. 7.** Effect of TEI-9647 on p21WAF1,CIP1 gene expression. HL-60 cells were treated in the absence (−) or presence (+) of 10–7 M TEI-9647 or 10–8 M 1α,25(OH)2D3 for the indicated time. Total RNA was extracted, and reverse transcription PCR of p21WAF1,CIP1 or β-actin was done as described under “Experimental Procedures.”
HL-60 cells. In contrast, neither analogue 1α,25(OH)2D3 was initially deactivated and metabolized to 1α,24R,25-trihydroxyvitamin D3 (1α, 24R,25(OH)3D3) by C-24 hydroxylation through a side-chain oxidation pathway resulting in C-23–C-24 cleavage, ultimately yielding 24,25,26,27-tetranor-1α,23-dihydroxyvitamin D3 (24,25,26,27-tetranor-1α,23(OH)3D3) in HL-60 cells (39–41). Moreover, 1α,25(OH)2D3 causes the expression of the 25(OH)D3-24-hydroxylase gene through VDR/DRE-mediated genomic action in various cells (42, 43). Because of a concern that the apparent inhibition of 1α,25(OH)2D3 might be a consequence of enhanced catabolism of 1α,25(OH)2D3, we have investigated the effect of the 1α,25-lactone analogues on the metabolism of 10−8 M [3H]1α,25(OH)2D3 in HL-60 cells (Fig. 9). In a control experiment in the absence of antagonist, 1α,25(OH)2D3 was metabolized to 1α,24R,25(OH)3D3, 24-oxo-1α,25-dihydroxyvitamin D3 (24-oxo-1α,25(OH)3D3), and 24,25,26,27-tetranor-1α,23(OH)3D3 at 8 h after incubation with HL-60 cells (data not presented), and the concentration of the 1α,25(0H)2D3 was markedly decreased by 24 h. The amounts of the 1α,25(OH)2D3 metabolites reached maximum levels 48–72 h after the cultivation. When 10−7 M TEI-9647 was added to the above culture system, it significantly inhibited the metabolism of [3H]1α,25(OH)2D3. The same is also true for TEI-9648, but the inhibitory action of TEI-9648 was stronger than that of TEI-9647. On the other hand, TEI-9616 and (23S,25R)-1α,25(OH)2D3-26,23-lactone did not inhibit the metabolism of 1α,25(OH)2D3. Collectively the results suggest that the antagonistic actions of TEI-9647 and TEI-9648 clearly occur despite blocking the metabolism of 1α,25(OH)2D3.

Many reports have described that the activation mechanism of steroid nuclear receptor families’ function involves complex formation with partner proteins after ligand/receptor binding (44–46). For example, VDR-retinoid X receptor complex formation is thought to be essential for initiating 1α,25(OH)2D3 responses (47). A possible consequence of TEI-9647 and TEI-9648 action may be to prevent heterodimer complex formation or the recruitment by the VDR receptor co-activator proteins like NCoA-62 (48) or steroid co-activator-1 (49). At present, we are carrying out further studies concerning the mode of action of TEI-9647 and TEI-9648.

Norman et al. (13, 15) reported that 1β,25(OH)2D3 acts as an antagonist of vitamin D3-induced nongenomic action. In these reports, 1β,25(OH)2D3 suppressed up-regulation of calcium transport in intestinal epithelium (transcalcitachia) (15) and stimulation of whole cell chloride currents in osteoblastic ROS 17/2.8 cells briefly exposed to 1α,25(OH)2D3 (13). In contrast, 1β,25(OH)2D3 did not antagonize HL-60 cell differentiation induced by 1α,25(OH)2D3, which is thought to be a genomic action of vitamin D3 (data not presented and Ref. 15). Considering these data, 1β,25(OH)2D3 would not be an antagonist of VDR/DRE-mediated genomic action of 1α,25(OH)2D3, but of nongenomic actions. The action spectrum of our novel antagonists, TEI-9647 and TEI-9648, is quite different from that of 1β,25(OH)2D3, from which we conclude that they are antagonists of 1α,25(OH)2D3-induced genomic action. It is not yet clear whether the lactones may function as antagonists of 1α,25(OH)2D3-mediated rapid nongenomic actions.

The biological significance of the natural (23S,25R)-1α,25(OH)2D3-26,23-lactone is not yet fully understood, though it is a major metabolite of 1α,25(OH)2D3 under physiological conditions (50). The metabolic pathways leading to 1α,25-lac-
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tone production from 1α,25(OH)2D3 are well investigated (19), whereas the further metabolism of 1α,25-lactone is not entirely known. It has been previously reported that the 25-dehydration reaction of 1α,25(OH)2D3 can occur in vivo, resulting in the production of both 24-dehydro-1α-hydroxvitamin D3 and 25-dehydro-1α-hydroxvitamin D3 (51). In the case of the naturally occurring 1α,25-lactone, a similar 25-dehydration reaction may possibly take place resulting in the production of TEI-9647. If our hypothesis is true, TEI-9647 should be present under physiological conditions and could possibly act as a negative regulator of hormonal action of 1α,25(OH)2D3 in vivo. We are now trying to identify further metabolites of the natural 1α,25-lactone in vivo and determining whether they include TEI-9647.

In conclusion, our data strongly suggest that the novel 1α,25-lactone analogues, TEI-9647 and TEI-9648, may be antagonists of VDR/DRE-mediated genomic actions. They are the first an-

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