Two Novel Metabolic Pathways of 22-Oxacalcitriol (OCT)

C-25 DEHYDRATION AND C-3 EPIMERIZATION AND BIOLOGICAL ACTIVITIES OF NOVEL OCT METABOLITES

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22-Oxacalcitriol (OCT) is an analog of calcitriol, characterized by potent differentiation-inducing activity and low calcemic liability. The metabolism of OCT has been studied and its polar metabolites, such as 24/26-hydroxylated-OCT and hexanor-1α,20-dihydroxyvitamin D3 (1α,20(OH)2D3), have been identified. In contrast, little is known about the less polar metabolites of OCT, which have been found in relatively large amounts. In this study, the in vitro metabolism of OCT was studied in UMR 106, Caco-2, and LLC-PK1 cells to identify the less polar metabolites and to assess their biological activity. OCT was initially metabolized to three less polar metabolites, 3-epi-OCT and two dehydrates, 25-dehydroxy-25-en-22-oxa-1α(OH)D3 (25-en-22-oxa-1α(OH)D3) and 25-dehydroxy-24-ene-22-oxa-1α(OH)D3 (24-ene-22-oxa-1α(OH)D3). We also observed further metabolites, the two C-3 epimers of the C-25 dehydrates, 25-ene-3-epi-22-oxa-1α(OH)D3 and 24-ene-3-epi-22-oxa-1α(OH)D3. The structures of these metabolites were successfully assigned by 1H NMR and LC-MS analyses. The three cell lines differ in their ability to metabolize OCT through the C-3 epimerization or the C-25 dehydration pathway. The biological activity of the OCT metabolites assessed by a luciferase reporter gene transcriptional activation system, binding assays for the vitamin D receptor (VDR) and vitamin D-binding protein (DBP), and assays for regulatory activities of cell differentiation and proliferation was found to be lower than that of OCT. Thus, both the C-3 epimerization and C-25 dehydration may work to reduce the biological activity of OCT.

The hormonal form of vitamin D3, 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3 or calcitriol)1 plays a crucial role in the regulation of calcium metabolism but it also regulates cell growth and differentiation in a variety of normal and malignant cells (1–3). Despite the potency of 1α,25(OH)2D3 as a negative growth regulator of colon (4, 5), breast (6, 7), and prostate cancer cells (8), its hypercalcemic properties have precluded its therapeutic use. To overcome this problem, vitamin D analogs with enhanced differentiation/anti-proliferative and reduced hypercalcemic properties have been synthesized. OCT is a synthetic analog, which has an oxygen atom at position 22, and has received government approval for use as an agent for the treatment of secondary hyperparathyroidism and psoriasis in Japan. OCT is rapidly cleared from the circulation due to its extremely low affinity for DBP (9, 10), and binds the chicken vitamin D receptor (VDR) with an approximately 8-fold lower affinity than 1α,25(OH)2D3 (11). However, OCT inhibits growth of psoriatic fibroblasts and enhances the immune response more effectively than 1α,25(OH)2D3 (12, 13). In contrast, OCT has reduced calcemic effects both in terms of mobilizing calcium from bone and in stimulating intestinal calcium transport in vitamin D-deficient and normal rats (14, 15). Recently, Kato and co-workers (16) reported that OCT induced interaction of the VDR with a transcriptional factor TIF-2, but not with other transcriptional factors such as SRC-1 and AIB-1, while 1α,25(OH)2D3 induced interactions of the VDR with all of the three co-factors tested. These factors such as metabolic clearance, tissue-specific distribution, cellular uptake, intracellular metabolism, and transcriptional regulation could contribute to differences in biological activity.

Recently, the metabolism of OCT has been studied in primary parathyroid cells (17) and keratinocytes (18) as well as osteosarcoma, hepatoma, and keratin cell lines (19). In all these systems, OCT is degraded into hydroxylated and side-chain truncated metabolites, 1α,20(OH)D3 and hexanor-20-oxo-1α-hydroxyvitamin D3 (20-oxo-1α(OH)D3). However, despite relatively large amounts of products from OCT (19), the structures and properties of the less polar metabolites have not yet been clarified. In the case of 1α,25(OH)2D3, one of these less polar metabolites has been identified as 3-epi-1α,25(OH)2D3, in which a hydroxy group at C-3 of the A-ring is epimerized from the β to the α position (20, 21). The C-3 epimerization of 1α,25(OH)2D3 occurs in vitro (20–22) and in vivo (23), and is a highly tissue-specific/cell differentiation-dependent process.

1 The abbreviations used are: 1α,25(OH)2D3, 25-hydroxyvitamin D3; 22-oxacalcitriol (22-oxa-1α,25-dihydroxyvitamin D3); 1α,20(OH)D3, hexanor-1α,20-dihydroxyvitamin D3; 24-ene-22-oxa-1α(OH)D3, 25-dehydroxy-24-ene-22-oxa-1α-hydroxyvitamin D3; VDR, vitamin D receptor; DBP, vitamin D-binding protein (Gc-globulin); HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; FCS, fetal calf serum.

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1 The abbreviations used are: 1α,25(OH)2D3, 25-hydroxyvitamin D3; 22-oxacalcitriol (22-oxa-1α,25-dihydroxyvitamin D3); 1α,20(OH)D3, hexanor-1α,20-dihydroxyvitamin D3; 24-ene-22-oxa-1α(OH)D3, 25-dehydroxy-24-ene-22-oxa-1α-hydroxyvitamin D3; VDR, vitamin D receptor; DBP, vitamin D-binding protein (Gc-globulin); HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; FCS, fetal calf serum.

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There are multiple aims of this study as follows: to identify the less polar metabolites of OCT including 3-epi-OCT in a rat osteosarcoma cells (UMR 106 cells); to investigate the further metabolism of OCT metabolites; to compare the production rate of metabolites and the OCT homologous human colon carcinoma cells (Caco-2) and porcine kidney cells (LLC-PK1); and to examine the biological activity of OCT metabolites. Our findings provide clear evidence that OCT is metabolized to at least three, and possibly five, less polar metabolites through two novel pathways, namely the C-3 epimerization and C-25 dehydroxylation pathways in addition to the well-known C-23/C-24/C-26 hydroxylation pathways in target cells.

EXPERIMENTAL PROCEDURES

Materials—OCT and its putative metabolite 24-ene-22-oxa-1α(3H)D3 were synthesized by Kuboder a et al. of Chugai Pharmaceutical Co., Ltd., Japan. The synthesis and unambiguous stereochromic assignment of 24-ene-22-oxa-1α(3H)D3 will be described elsewhere. 3-epi-OCT and 3-epi-1α,25(OH)2D3 were synthesized by Hatakeyama et al. of Nagasaki University, Japan. 1α,25(OH)2D3 and 25-hydroxvitamin D3 (25(OH)D3) were obtained from Solvay-Duphar Co. (Weesp, The Netherlands). 1α,25(OH)2D3[26,27-3H]-HID (6.6 TBq/mmol) and 25(OH)D3[26,27-3H] (3.9 TBq/mmol) were purchased from American Biosciences. Culture media and antibiotics were purchased from Invitrogen. Deuterated chloroform (CDCl3, 99.9%, NMR analytical grade) was purchased from EURISO-TOP (Gif-Sur-Yvette, France). Organic solvents of HPLC grade were obtained from Wako Pure Chemical Industries, Ltd.

Culture—UMR 106, Caco-2, and LLC-PK1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). UMR 106 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Caco-2 cells were maintained in Eagle's Medium containing 10% FCS and 1% non-essential amino acids. LLC-PK1 cells were maintained in Medium 199 containing 10% FCS and 1% bovine serum albumin, in buffered saline without calcium and magnesium (PBS). All culture media contained penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 in air, and the medium was changed every three days. In the experiments described below, OCT or 3-epi-OCT was added to culture medium in ethanolic solution, the final ethanol concentration in the medium never exceeding 0.1% (v/v).

Generation of OCT and 3-epi-OCT Metabolites—For structure assignments, 10 plates of UMR 106 cells cultured in 150-mm culture dishes were used. Monolayers were washed with 10 ml of phosphate-buffered saline without calcium and magnesium (PBS) and then incubated in 10 ml of media containing 1% bovine serum albumin, in PBS modified with charcoal (0.025% dextran T-150/0.25% Norit A charcoal decolorizing solution). Following centrifugation, 1.0 ml of each supernatant was collected and transferred into a scintillation vial to measure radioactivity. Competitive displacement of 25(OH)2[3H]-D3 from vitamin D-deficient rat serum DBP by OCT and its metabolites was determined under equilibrium ligand-binding conditions (19). A total of 82 fmol of 25(OH)2[3H]-D3, in 50 μl of ethanol was mixed with increasing amounts of 25(OH)D3 (0.00125–16 ng/tube), 1α,25(OH)2D3 (0.04–4000 ng/tube) or OCT metabolites (0.06–1000 ng/tube) in 100 μl of ethanol. Next, 1 ml of vitamin D-deficient rat serum diluted 1:7,000 with freshly prepared barbital acetate buffer (3.5 mM acetic acid, 3.5 mM sodium barbiturate, 0.13 mM NaCl, 0.1% ovalbumin, pH 8.6) was added, and the samples were incubated for 1 h at 20 °C. Following centrifugation, 1.0 ml of each supernatant was collected and transferred into a scintillation vial to measure radioactivity.

Transfection and Luciferase Activity Assay—The human osteosarcoma cell line MG-63 (ATCC) was maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% dextran-coated charcoal-treated FCS. Cells (2 × 10⁴) were suspended in 2 ml of the medium and transfected with 0.5 μg of luciferase reporter plasmid (pGVB2 vector, Toyo Ink Co., Ltd., Japan) carrying a human osteocalcin gene promoter (100 ng) including the VDRRE (28) or a rat CYP24 gene promoter (~291/9) including the two VDREs (29), and 0.25 μg of the pRL-CMV vector (pgVB2 vector, Toyo Ink Co., Ltd.) as an internal control. The transfection agent used was Tfx-50 reagent (Promega Corp. Madison, WI). The cells were incubated with 10 μl or 10 μl of 1α,25(OH)2D3 or OCT for 48 h. The luciferase activity in the culture media was measured with a luciferase assay system (Toyo Ink Co., Ltd.). Transfection-activation measured by luciferase activity was standardized with the luciferase activity of the same cells determined by the Sea Panys Luciferase assay system as a control (Toyo Ink Co., Ltd.). Each set of experiments was repeated at least three times.
charcoal-treated FCS and kanamycin (0.06 mg/ml) at 37 °C in a humidified atmosphere of 5% CO₂ in air. For synchronization at the S phase, cells (4 × 10⁶) were cultured in 30 ml of RPMI 1640 medium for 24 h and subsequently cultured for 16 h in RPMI 1640 medium supplemented with 2.5 mM thymidine. After washing with PBS(−) twice, the cells were cultured in normal medium for 10 h and then in 2.5 mM thymidine medium for 16 h. The cells thus obtained were used for flow cytometry. The cells (10⁶ cells/well) were placed in 24-well tissue culture plates and cultured for 3 days with OCT or its metabolites (10⁻⁶, 10⁻⁵ M). Each group of cells was washed with PBS(−) and re-suspended in PBS(−) containing 0.2% Triton X-100 and 100 µg of RNase, then incubated at 37 °C for 1 h. Cells were washed with PBS(−) and incubated with 0.5 ml of DNA-staining solution containing propidium iodide (50 µg/ml) at 4 °C for 20 min. The cells were analyzed with a flow cytometer equipped with an argon laser (488 nm, Becton Dickinson FACScan™), and cell cycle distribution was analyzed by ModiFiT LT (Verity). For analysis of cell cycle profiles, at least three experiments were repeated at least three times.

Statistics—Values were calculated as means ± S.E. Significance levels were determined by Student’s t test.

RESULTS

Metabolism of OCT in UMR 106 Cells

Incubation of 10 µM OCT with UMR 106 cells for 48 h resulted in the formation of 5 metabolites (Fig. 1A), all of which demonstrated the typical vitamin D chromophore (λ_max of 265 nm, λ_min of 228 nm; data not shown). These metabolites were not formed when OCT was incubated with medium in the absence of cells, or when cells were incubated with medium alone (data not shown). 2 of 5 metabolites, labeled Metabolites 4 and 5, were polar metabolites of OCT and identical to the previously identified 1α,20(OH)₂D₃ and 24-hydroxy-OCT (24(OH)/OCT), respectively (19). Three other less polar metabolites, labeled Metabolites 1, 2, and 3 have not been isolated and identified to date and were thus purified by extensive rechromatography twice on Zorbax CN and Zorbax SIL for structure assignments.

Identification of Less Polar Metabolites of OCT (Metabolites 1, 2, and 3)

OCT Metabolite 1—The 1H chemical shifts and coupling constants assigned by one-dimensional and two-dimensional COSY and NOESY spectra of Metabolite 1 are summarized in Table I. The most pronounced differences between the 1H NMR spectra of Metabolite 1 and OCT were found in the 1.6–1.8 ppm and 4.6–4.8 ppm regions. The disappearance of one of the singlets from the methyl protons in position 26 or 27 (observed at 1.21 and 1.23 ppm in OCT) and the appearance of two singlets at 4.70 and 4.74 ppm, which were assigned to protons of the exo-methylene group, were observed. These findings indicate that dehydration took place at the C-25 hydroxyl group. The resonances from H-24 (2.23 ppm) were shifted downfield compared with those of OCT (1.71 ppm). In the two-dimensional COSY spectrum, cross-peaks of the neighboring protons were detected. Connectivity of the H-23 and 23′ (3.33 and 3.63 ppm) to the H-24 resonance (2.23 ppm) was observed. The other cross-peaks observed in two-dimensional COSY analysis are shown in Fig. 2A. Metabolite 1 was assigned to 25-ene-22-oxa-1α(OH)D₃, which has an OCT-like structure with an additional exo-methylene group at the end of the side-chain as a result of dehydration at the C-25 hydroxyl group of OCT. In the LC-MS spectrum of Metabolite 1, [M + NH₄]⁺ was observed at m/z 418.6 and indicated a reduction of 18 mass units from OCT (Table I). Metabolite 1 gave peaks at m/z 401.6 [M + H]+ and 423.7 [M + Na]+. Therefore, the LC-MS spectrum supports the assignment of Metabolite 1 structure by 1H NMR analysis.

OCT Metabolite 2—The 1H NMR spectrum of Metabolite 2 also showed an unchanged structure of ring A, containing the triene system (Table I). The resonance from H-24 (5.32 ppm) was changed in contrast with that of OCT (1.71 ppm). The intensity of the H-24 signal was found to be only half of the expected value. Two singlets from the methyl protons in positions 26 and 27 (1.64 and 1.71 ppm) were shifted downfield compared with those of OCT (1.21 and 1.23 ppm). The resonances from H-23 and 23′ (3.79 and 4.01 ppm) were also shifted downfield. These findings indicate that an olefin group was introduced between C-24 and C-25, and only one proton atom at H-24 remained in Metabolite 2. In the two-dimensional COSY spectrum, cross-peaks showing connectivity between the neighboring protons were clearly observed (Fig. 2B). The signal at 1.71 ppm was assigned to the proton of trans position against C-23 (namely, C-26 position) because of the presence of cross-peaks with H-23′ and 24 that demonstrate long range coupling. The signal at 1.64 ppm was also assigned to the proton of cis position against C-23 (namely, C-27 position). The LC-MS spectrum of Metabolite 2 in principle showed the same features as the spectrum of Metabolite 1; [M + NH₄]⁺ was observed at m/z 418.6, suggesting a reduction of 18 mass units from OCT (Table I). Consequently, Metabolite 2 was assigned to 24-ene-22-oxa-1α(OH)D₃, a dehydrate of OCT in which a double bond was
TABLE I

1H-NMR and LC-MS analyses of OCT and 3-epi-OCT metabolites

| Compound          | 1H-NMR analysis, 1H chemical shifts and coupling constants (chemical shifts: ppm, coupling constants: Hz) | LC-MS analysis, characteristic ions |
|-------------------|-------------------------------------------------------------------------------------------------------------|-------------------------------------|
| Synthetic standards |                                                                                                            |                                     |
| OCT               | (1H, m) (1H, m) (1H, d, J = 11.5) (1H, d, J = 11.0) (1H, m) (1H, m) (1H, m) (1H, td, J = 5.5, 9.5) (1H, td, J = 5.0, 9.5) (2H, t, J = 5.5) | (3H, s) (3H, s) 441.7 [M + Na]⁺ |
| 3-epi-OCT         | (1H, m) (1H, m) (1H, d, J = 12.0) (1H, d, J = 11.5) (1H, m) (1H, m) (1H, m) (1H, td, J = 5.5, 9.0) (1H, td, J = 6.0, 9.0) (2H, t, J = 5.5) | (3H, s) (3H, s) 419.6 [M + H]⁺ |
| 24-ene-22-oxa-1α/2β-OH-D₂ | (1H, m) (1H, m) (1H, d, J = 11.5) (1H, d, J = 11.5) (1H, m) (1H, m) (1H, m) (1H, dd, J = 7.0, 11.0) (1H, dd, J = 6.5, 11.0) (1H, m) | (3H, s) (3H, s) 418.6 [M + H]⁺ |
| OCT Metabolites   |                                                                                                            |                                     |
| Metabolite 1      | (1H, m) (1H, m) (1H, d, J = 11.0) (1H, d, J = 11.0) (1H, m) (1H, m) (1H, m) (1H, td, J = 7.5, 9.0) (1H, td, J = 7.0, 9.0) (2H, td, J = 6.0, 6.3) | (3H, s) (3H, s) 4237 [M + Na]⁺ |
| Metabolite 2      | (1H, m) (1H, m) (1H, d, J = 11.0) (1H, d, J = 11.0) (1H, m) (1H, m) (1H, m) (1H, td, J = 7.5, 9.0) (1H, td, J = 7.0, 9.0) (2H, td, J = 6.0, 6.3) | (3H, s) (3H, s) 4186 [M + NH₄]⁺ |
| Metabolite 3      | (1H, m) (1H, m) (1H, d, J = 11.5) (1H, d, J = 11.5) (1H, m) (1H, m) (1H, m) (1H, td, J = 7.0, 11.5) (1H, m) (3H, s) (3H, s) 4186 [M + H]⁺ |
| 3-epi-OCT Metabolites |                                                                                                        |                                     |
| Metabolite 1'     | (1H, m) (1H, m) (1H, d, J = 11.5) (1H, d, J = 11.5) (1H, m) (1H, m) (1H, m) (1H, td, J = 7.5, 9.0) (1H, td, J = 6.5, 9.0) (2H, td, J = 5.0, 6.5) | (3H, s) (3H, s) 4186 [M + Na]⁺ |
| Metabolite 2'     | (1H, m) (1H, m) (1H, d, J = 11.5) (1H, d, J = 11.0) (1H, m) (1H, m) (1H, m) (1H, td, J = 7.0, 11.5) (1H, m) (3H, s) (3H, s) 4186 [M + H]⁺ |
Two Novel Metabolic Pathways of OCT

Two-dimensional COSY spectrum of OCT Metabolites 1 and 2 recorded in CDCl₃. A, metabolite 1; B, metabolite 2.

Introduction of cross-peaks in Metabolite 1 and Metabolite 2.

Spectral analyses were performed. In the 1H NMR spectrum of oxa-1-22-oxa-1 recorded in CDCl₃.

Metabolite 2. Metabolite 2 co-migrated with authentic 24-ene-22-olite 1. 1H NMR and LC-MS spectra of synthesized 24-ene-22-olite 1.

OCT Metabolite 3—Metabolite 3 was inferred to be 3-epi-OCT by co-migration with the authentic standard in HPLC analysis. To confirm this interpretation, 1H NMR and LC-MS analyses were performed.

OCT Metabolite 3 is a diastereomer or a geometric isomer of OCT. Based on the findings of HPLC, 1H NMR, and LC-MS analyses, Metabolite 3 was assigned as 3-epi-OCT, which has a changed configuration of a hydroxyl group at C-3 of the A-ring.

Metabolism of 3-epi-OCT in UMR 106 Cells

Incubation of UMR 106 cells with 10 µM 3-epi-OCT for 48 h resulted in the formation of 4 metabolites (Fig. 1B). Two of 4 metabolites, labeled Metabolites 3' and 4' were corresponded to the C-3 epimers of 1α,20(OH)D₃ and 24(OH)OCT, respectively. Two other less polar metabolites, labeled Metabolites 1' and 2' were purified for structure assignments by 1H NMR spectroscopy and LC-MS analyses.

Identification of Less Polar Metabolites of 3-epi-OCT (Metabolites 1' and 2')

The proton chemical shifts assigned by one- and two-dimensional COSY and NOESY spectra of purified Metabolites 1' and 2' were summarized in Table I. Except for the chemical shifts of H-1 and 3, the resonances from all protons of Metabolites 1' and 2' matched those of Metabolites 1 and 2, respectively. The chemical shifts of H-1 and 3 of Metabolites 1' and 2' were observed at the same upfield position as 3-epi-OCT compared with OCT. In the LC-MS spectra of Metabolites 1' and 2', [M+NH₄]⁺ was also observed at m/z 418.6, and the other ions, 401.6 [M+H]⁺ and 423.7 [M+Na]⁺ showed identical patterns to the spectra of Metabolites 1 and 2 (Table I). From the findings of 1H NMR and LC-MS analyses, Metabolites 1' and 2' were assigned as 25-ene-3-epi-22-oxa-1α(OH)D₃ and 24-ene-3-epi-22-oxa-1α(OH)D₃, respectively. In this experiment, OCT was not detected as a metabolite of 3-epi-OCT. This finding suggests that C-3 epimerization occurs unidirectionally as previously reported (20, 31).

Production Rates of OCT Metabolites in UMR 106, Caco-2 and LLC-PK₁ Cells

Because of a lack of authentic compounds for 25-ene-22-oxa-1α(OH)D₃ and 24(OH)OCT, the amounts of these metabolites were measured by Zorbax SIL HPLC using the metabolites purified from UMR 106 cell culture as the standard compounds. The same less polar metabolites of OCT were generated in all cell lines tested, although there were differences existing in the amounts of products formed among cell types (Fig. 3). The major metabolite found in cell cultures of Caco-2 and LLC-PK₁ was 24(OH)OCT, whereas 25-ene-22-oxa-1α(OH)D₃ appeared to be more prevalent in UMR 106 cells. Interestingly, in UMR 106 cells the production ratio of 25-ene-22-oxa-1α(OH)D₃ to 24-ene-22-

Fig. 2. Two-dimensional COSY spectrum of OCT Metabolites 1 and 2 recorded in CDCl₃. A, metabolite 1; B, metabolite 2.

Fig. 3. Relative generated amounts of OCT metabolites in UMR 106, Caco-2, and LLC-PK₁ cells. Each cell line was incubated with 10 µM OCT for 48 h. The results are expressed as the total amount of product formed in nmol/plate/48 h and represent the mean of three experiments (values in column).
Fig. 4. Dose response and time course of OCT or 3-epi-OCT metabolism in UMR 106 cells. A, amounts of OCT metabolites in UMR 106 cells incubated with 0.1–10 μM OCT for 24 h. B, amounts of OCT metabolites in UMR 106 cells incubated with 5 μM OCT for 1–48 h. ■, 25-ene-22-oxa-1α(OH)D_{3}; □, 24-ene-22-oxa-1α(OH)D_{3}; ●, 3-epi-OCT; ●, 24(OH)OCT. C, amounts of the less polar metabolites of 3-epi-OCT in UMR 106 cells incubated with 0.1–10 μM 3-epi-OCT for 24 h. D, amounts of the less polar metabolites of 3-epi-OCT in UMR 106 cells incubated with 5 μM 3-epi-OCT for 1–48 h. The results represent the mean of three experiments (values in column). ■, 25-ene-3-epi-22-oxa-1α(OH)D_{3}; □, 24-ene-3-epi-22-oxa-1α(OH)D_{3}.

Dose Response and Time Course Studies of Metabolism of OCT and 3-epi-OCT in UMR 106 Cells

25-Ene-22-oxa-1α(OH)D_{3}, 24-ene-22-oxa-1α(OH)D_{3}, 24(OH)OCT, and 3-epi-OCT were produced in a dose-dependent manner in up to 10 μM OCT as shown in Fig. 4A. When UMR 106 cells were incubated with 0.1 μM OCT, 24(OH)OCT was predominantly produced. However, when the cells were incubated with 1–10 μM OCT, the major metabolite was 25-ene-22-oxa-1α(OH)D_{3}. Interestingly, the production ratio of 25-ene-22-oxa-1α(OH)D_{3} to 24-ene-22-oxa-1α(OH)D_{3} was 2:1 at any concentration of OCT. In a time course study, 25-ene-22-oxa-1α(OH)D_{3} and 24(OH)OCT were first detected approximately 1 h after the incubation was begun, and continued to increase up to the end of the incubation period (Fig. 4B). 24-ENE-22-oxa-1α(OH)D_{3} and 3-epi-OCT were first apparent at 3 h of incubation. 24-ENE-22-oxa-1α(OH)D_{3} continued to increase up to the end of the incubation period, whereas 3-epi-OCT gradually increased and reached only 30% of the amount of 25-ene-22-oxa-1α(OH)D_{3} by the end of the incubation period.

We also examined dose response and time course studies of 3-epi-OCT metabolism in UMR 106 cells. In a dose response study, two dehydrates were produced in a dose-dependent manner in up to 10 μM 3-epi-OCT (Fig. 4C). At any concentration, the ratio of 25-ene-3-epi-22-oxa-1α(OH)D_{3} to 24-ene-3-epi-22-oxa-1α(OH)D_{3} was exactly 2:1. In a time course study, the amounts of the two dehydrates continued to increase up to the end of the incubation period (Fig. 4D).

VDR and DBP Binding Properties of OCT and Its Less Polar Metabolites

All of the metabolites tested had a lower binding affinity for VDR than OCT (Fig. 5A). Relative binding affinities calculated for 50% displacement of 1α,25(OH)_{2}[3H]D_{3} were only 0.13, 0.30, and 0.47% of OCT for 25-ene-22-oxa-1α(OH)D_{3}, 24-ene-22-oxa-1α(OH)D_{3}, and 3-epi-OCT, respectively. Fig. 5B shows the rat plasma DBP binding assay with the metabolites in comparison with 1α,25(OH)_{2}D_{3} and OCT. All of the metabolites tested had an extremely low binding affinity for DBP, like OCT.

Fig. 5. Relative binding affinity of OCT, novel OCT metabolites, and vitamin D metabolites for VDR and DBP. A, displacement curves of 1α,25(OH)_{2}[3H]D_{3} from the VDR by the vitamin D derivatives. The binding affinity was compared by the concentration of each compound needed to achieve 50% displacement of 1α,25(OH)_{2}[3H]D_{3} from thymus VDR. B, competitive displacement of 25(OH)[3H]D_{3} from vitamin D-deficient rat plasma DBP with the vitamin D derivatives. The binding affinity was compared by the concentration of each compound needed to achieve 50% displacement of 25(OH)[3H]D_{3} from DBP. ●, OCT; ○, 3-epi-OCT; ■, 25-ene-22-oxa-1α(OH)D_{3}; □, 24-ene-22-oxa-1α(OH)D_{3}; ▲, 1α,25(OH)_{2}D_{3}; △, 25(OH)D_{3}. 

oxa-1α(OH)D_{3} was 2:1; however, in Caco-2 and LLC-PK1 cells, the production ratios of 25-ene-22-oxa-1α(OH)D_{3} to 24-ene-22-oxa-1α(OH)D_{3} were 1:4 and 1:11, respectively.

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Target Gene Activation by OCT and its Less Polar Metabolites

The transcription-inducing activities of OCT metabolites on a human osteocalcin gene promoter in MG-63 cells are shown in Fig. 6A. At $10^{-8}$ M, the transcriptional activities were about 8, 24, and 33% of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$, and 3-epi-OCT, respectively. Similarly, the transcription-inducing activities of OCT metabolites on a rat CYP24 gene promoter in transfected MG-63 cells are shown in Fig. 6B. At $10^{-8}$ M, the transcriptional activities were about 2, 11, and 15% of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$, and 3-epi-OCT, respectively. When we examined the transcriptional activities of the metabolites on a human osteocalcin gene promoter and a rat CYP24 gene promoter in transfected UMR 106 cells, we observed the same induction levels for the two genes as observed in transfected MG-63 cells (data not shown). Thus, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT were found to be less active than OCT, with potencies between one-third and one-tenth in terms of the activation of vitamin D target genes.

Anti-proliferative and Differentiation-inducing Activities of OCT and Its Less Polar Metabolites

At $10^{-8}$ M, no metabolites of OCT showed significant activity of arresting the cell cycle at G$_0$-G$_1$ phase as compared with 1α,25(OH)$_2$D$_3$ and OCT (Fig. 7A). Three OCT metabolites had little inducing effect on cell surface CD11b antigen expression in a human promyelocytic leukemia cell line, HL-60 (Fig. 7B). At $10^{-8}$ M, the biological activities were only 8, 8, and 12% of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$, and 3-epi-OCT, respectively.

DISCUSSION

We were able to detect several distinct peaks ascribed to less polar metabolites of OCT in sufficient quantities to assign structures using $^1$H NMR and LC-MS techniques. The formation of these less polar metabolites was shown to be an enzymatic process, because the metabolites were not observed in no-cell controls incubated with OCT. The novel less polar metabolites identified include two dehydrates (25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$, and 3-epi-OCT). In addition, we demonstrated that the 3-epi-OCT was also converted into two dehydrates (25-ene-3-epi-OCT and 24-ene-3-epi-OCT). These findings clearly indicate that UMR 106 cells are able to metabolize OCT to its less polar metabolites both via the C-25 dehydration and C-3 ep-
merization pathway (Fig. 8). To the best of our knowledge, this is the first definite structural assignment of the C-25 dehydrates of OCT. Siu-Caldera et al. (22) studied the metabolism of 1α,25(OH)₂D₃ in UMR 106 and ROS 17/2.8 cells and found a peak corresponding to a less polar metabolite (denoted as M1). Due to the insufficient quantity, its definite structural assignment was not established. If this metabolite M1 is one of the two dehydrates of 1α,25(OH)₂D₃, namely 25-ene-1α-hydroxyvitamin D₃ or 24-ene-1α-hydroxyvitamin D₃, then C-25 dehydration would likely be a common metabolic pathway of both 1α,25(OH)₂D₃ and synthetic analogs.

Two possible metabolic routes from OCT to dehydrates can be considered; one via the direct C-25 dehydration, and the other via the C-24 and C-26 hydroxylation. The latter route via the CYP24 metabolic pathway seems unlikely because the dehydration of hydroxyl groups at positions C-24/C-25 or C-25/C-26 has not been found in the metabolism of steroid hormones. It is generally accepted that 24(OH)OCT is metabolized to 24-oxo-OCT, and that 26-hydroxy-OCT (26(OH)OCT) is likely to be metabolized to 23,26-dihydroxy-OCT. Therefore, both 24(OH)OCT and 26(OH)OCT seem unlikely to be precursors of the two OCT dehydrates. The former route is more plausible. Both the 24-ene-22-oxa-1α(OH)D₃ and the 25-ene-22-oxa-1α(OH)D₃ can be formed from OCT by enzymatic C-25 dehydroxylation followed by dehydrogenation at the positions C-24 and C-26, respectively. If the latter oxidation occurs non-enzymatically, then the production of 24-ene-22-oxa-1α(OH)D₃ is expected to be greater than that of 25-ene-22-oxa-1α(OH)D₃ on the basis of the chemical reaction. In this study, unexpectedly, the production of 25-ene-22-oxa-1α(OH)D₃ was greater than that of 24-ene-22-oxa-1α(OH)D₃ in UMR 106 cells. Therefore, the C-25 dehydroxylation process of OCT is suspected to be under strict cell-specific control, or further metabolism of the dehydrates may differ with cell line. The findings of the biological studies here with two dehydrates of OCT demonstrated that their biological activities are considerably lower than OCT. Thus, it appears that like the C-23/C-24 hydroxylation pathways, the C-25 dehydroxylation pathway contributes to reducing the high potency of OCT.

In this study, we also have shown that OCT is converted into its C-3 epimer. The C-3 epimerization is a unique and important pathway because the resulting C-3 epimer may be metabolized under the same way as the parent OCT. Reddy et al. (31) recently demonstrated that 3-epi-1α,25(OH)₂D₃ was further metabolized via the C-23/C-24 oxidation pathways as 1α,25(OH)₂D₃. The C-3 epimerization is not specific to 1α,25(OH)₂D₃ and OCT. Higashi et al. identified 3-epi-24,25(OH)₂D₃-24-glucuronide in rat bile (32) and 3-epi-24,25(OH)₂D₃ in rat plasma (33). More recently, we reported a definite structural assignment for 3-epi-24,25(OH)₂D₃ isolated from a UMR 106 cell culture (27). In addition, Reddy et al. (34) demonstrated that synthetic vitamin D analogs, 1α,25(OH)₂-16-ene-23-yne-vitamin D₃ and 1α,25(OH)₂-16-ene-23-yne-20-epi-vitamin D₃ are metabolized to their respective C-3 epimers in UMR 106 cells. These findings clearly indicate that most of the vitamin D derivatives are metabolized through the C-3 epimerization pathway in vitro and in vivo. It is also interesting to note that the rate of C-3 epimerization varies depending upon the structure of vitamin D derivatives. We observed that the rate of C-3 epimerization of 1α,25(OH)₂D₃ was about 3-fold higher than that of OCT in UMR 106 cells. In addition, the rate of C-3 epimerization of 1α,25(OH)₂D₃ was about 2-fold higher than that of 24,25(OH)₂D₃ (27). Recently, Reddy et al. (34) reported that the rate of C-3 epimerization of 1α,25(OH)₂-16-ene-23-yne-D₃ was 10-fold lower than that of its C-20 epimer. These findings imply that the enzyme(s) responsible for C-3 epimerization can recognize structural differences not only in the A-ring but also in the side-chain of vitamin D derivatives. The C-3 epimerization pathway has been shown to be present in a variety of normal and malignant cells. However, its contribution to the metabolism of vitamin D appears to be relatively low compared with the C-23/C-24 oxidation pathways.

![Fig. 8. Novel metabolic pathway of OCT.](image-url)
Two Novel Metabolic Pathways of OCT

except for specific cell lines (e.g. UMR 106 cells). Rat osteosarcoma ROS 17/2.8 cells, in which the C-24 oxidation pathway is not active, has been shown to metabolize 1α,25(OH)2D3 via the C-3 epimerization pathway (22). In contrast, the perfused rat kidney and human promyelocytic leukemia cell line HL-60, in which the C-23/C-24 hydroxylation pathways are highly expressed, do not metabolize 1α,25(OH)2D3 to via the C-3 epimerization pathway (22, 35). These findings imply that C-3 epimerization pathway is cell-selective and contributes to the metabolism of vitamin D in concert with the C-23/C-24 hydroxylation pathways. It is interesting to note that 3-epi-1α,25(OH)2D3 was almost equipotent to 1α,25(OH)2D3 in suppressing parathyroid hormone secretion in bovine parathyroid cells (36) and in inhibiting keratinocyte proliferation (24, 37), expressing parathyroid hormone secretion in bovine parathyroid cells. The interplay of these metabolic pathways may be important in the regulation of OCT metabolism and its biological functions except for specific cell lines (e.g. UMR 106 cells).

In summary, we present evidence that a novel C-25 dehydroxylation pathway is involved in the metabolism of OCT. Furthermore, we also demonstrated that OCT is metabolized to 3-epi-OCT, and that the resulting 3-epi-OCT is further metabolized to two dehydroxyhydroxylation pathways. In UMR 106 cells, OCT is predominately metabolized via the C-25 dehydroxylation pathway. On the other hand, in Caco-2 and LLC-PK1 cells, OCT is predominately metabolized via the C-23/C-24 hydroxylation pathways. The interplay of these metabolic pathways may be important in the regulation of OCT metabolism and its biological functions in its target cells.

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