In Vitro Metabolism of the Vitamin D Analog, 22-Oxacalcitriol, Using Cultured Osteosarcoma, Hepatoma, and Keratinocyte Cell Lines*

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Using four cultured cell models representing liver, keratinocyte, and osteoblast, we have demonstrated that the vitamin D analog, 22-oxacalcitriol is degraded into a variety of hydroxylated and side chain truncated metabolites. Four of these metabolic products have been rigorously identified by high pressure liquid chromatography, diode array spectrophotometry, and gas chromatography-mass spectrometry analysis as 24-hydroxylated and 26-hydroxylated derivatives as well as the cleaved molecules, hexanor-1α,20-dihydroxyvitamin D3 and hexanor-20-oxo-1α,25-dihydroxyvitamin D3. Comparison with chemically synthesized standards has revealed the stereochemistry of the biological products. Although differences exist in the amounts of products formed with the different cell types, it is apparent that 22-oxacalcitriol is subject to metabolism by both vitamin D-inducible and noninducible enzymes. Time course studies suggest that the truncated 20-alcohol is derived from a side chain hydroxylated molecule via a hemiacetal intermediate and the 20-oxo derivative is likely formed from the 20-alcohol. Biological activity measurements of the metabolites identified in our studies are consistent with the view that these are catabolites and that the biological activity of 22-oxacalcitriol is due to the parent compound. These results are also consistent with recent findings of others that the biliary excretory form of 22-oxacalcitriol is a glucuronide ester of the truncated 20-alcohol.

It is now firmly established that the hormonal form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α, 25-(OH)2D3 or calcitriol) has potent cell differentiating/anti-proliferative activities in addition to its role in calcium homeostasis (1). This has led researchers in both universities and the pharmaceutical industry to search for so-called “noncalcemic” vitamin D analogs with accentuated differentiating/anti-proliferative properties and reduced ability to cause hypercalcemia (2, 3). 22-Oxacalcitriol (OCT) was an early analog developed for this purpose and contains a 22-oxygen atom that replaces the 22-carbon of calcitriol. OCT binds to the chicken vitamin D receptor (VDR) with an approximately 8 times lower affinity than 1α,25-(OH)2D3 (4). However, OCT is 10 times more effective than 1α,25-(OH)2D3 in suppressing cell growth and inducing differentiation of the mouse myelocytic leukemia cell, WEHI-3, in vitro (5). OCT also possesses an enhanced in vivo immunomodulatory potency in mice that is 50 times higher than that of 1α,25-(OH)2D3 (6). In contrast, OCT has reduced calcemic activity in vivo (7a–9a) that of 1α, 25-(OH)2D3, both in terms of mobilizing calcium from bone and in stimulating intestinal calcium transport in vitamin D-deficient and normal rats (7, 8). The mechanisms responsible for these differences in biological activity remain unclear, but factors such as cellular uptake and intracellular metabolism could contribute to these differences. OCT binds poorly to the vitamin D binding protein (DBP) and is transported in the plasma bound by lipoproteins (chylomicrons and low density lipoprotein) in vivo (9), and this leads to an unusual distribution pattern with a degree of concentration of the vitamin D analog in parathyroid tissue (10). In contrast, little is known about OCT metabolism except that it appears to be excreted as a glucuronide conjugate in the bile, possibly a derivative of a truncated version of OCT (10). Furthermore, there have been suggestions that a metabolite with a truncated side chain may be formed in bovine parathyroid cell cultures in vitro (11), although details of this have yet to be published.

In these studies we set out to provide convincing physicochemical identification for metabolites generated in a variety of cultured cell models, namely the human hepatoma lines HepG2 and Hep3B (12), the rat osteosarcoma cell line UMR-106 (13), and the human keratinocyte cell lines HPK1A and HPK1A ras (14, 15). Because these cell models mimic vitamin D metabolism found in vivo, we expected to observe the same metabolites found in vivo in some cases tentatively identified by others (10, 11). In addition, our objective was to study the rate of OCT metabolism in various cell lines in order to gauge the involvement of vitamin D-inducible catabolic pathways as compared with more general metabolizing systems. Our results support the concept that OCT is subject to extensive metabolism in a variety of tissues that leads to side chain truncated forms excreted in the bile.

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1 D3 and D2 indicate vitamins D3 and D2, respectively, and OH and (OH)2 indicate hydroxy and dihydroxy, respectively. Therefore, for example, 1α,25-(OH)2D3 represents 1α,25-dihydroxyvitamin D3.

2 The abbreviations used are: OCT, 22-oxacalcitriol; DBP, vitamin D binding protein (GC-globulin); HIM, hexane/isopropanol/methanol; VDR, vitamin D receptor; HPLC, high pressure liquid chromatography; GC-MS, gas chromatography-mass spectrometry; TMS, trimethylsilyl.
EXPERIMENTAL PROCEDURES

Materials

1α,25-(OH)2D3 was a generous gift from Dr. M. Uskokovic of Hoffmann-LaRoche. OCT was synthesized by the method of Murayama et al. (7). Putative metabolites of OCT, including 22,23,24,25,26,27-hexanor-1α,20-(OH)2D3 and 22,23,24,25,26,27-hexanor-20α-xo-1α-hydroxyvitamin D3 as well as 24- and 26-hydroxylated derivatives, were synthesized as recently described (16). 25-OH-D3 and (23,24n)-25-OH-D3 (3.33 TBq/mmol) were obtained from Du Pont Co. (Wespe, Holland) and Amersham Corp., respectively. Cell culture media and fine chemicals were purchased from Life Technologies, Inc. Fetal calf serum was from Flow Laboratories (Costa Mesa, CA). Derivatizing agents and solvents were purchased as follows: sodium periodate (BDH Chemicals Ltd, Poole, UK); sodium borodeuteride (Merck, Sharp, & Dohme, Montreal, PQ, Canada); N-trimethylsilylimidazole (Pierce); and HPLC grade organic solvents (Caledon, Ontario, Canada). All other reagents were analytical reagent grade.

Cell Culture

The cell lines UMR106 and HepG2 were cultured as described previously (12, 13). Immortalized (HPK1A) and transformed (HPK1A-ras) cell lines were established from normal human keratinoctyes by stable transfection with human papillomavirus type 16 followed by transformation with the activated H-ras oncogene, respectively (14, 17). HPK1A and HPK1A-ras cell lines were cultured as described previously (15).

Generation of OCT Metabolites

As described previously (15), 150-mm culture dishes containing monolayers (HPK1A, HPK1A-ras, UMR106, or HepG2) cultured to confluence in the presence of fetal calf serum were washed with phosphate-buffered saline and then incubated in Dulbecco’s minimal essential medium containing 1% bovine serum albumin in the presence of 41.8 μg of OCT (10 μM) for 48 h at 37°C. Except in the time course experiments described below, cells were not treated with 1α,25-(OH)2D3 to induce catabolic enzymes. No cell or dead cell controls consisted of 10 ml of medium or microwave-treated cells incubated with OCT under the same conditions for the same length of time. Medium and cells were removed for analysis of OCT metabolites, whereas representative plates of cells were used for determination of cell number using a model 2M automatic cell counter. In the time course study, HPK1A-ras cells were cultured to late log phase in Dulbecco’s minimal essential medium containing 10% fetal calf serum and treated with 10 ml 1α,25-(OH)2D3 for 16 h in order to maximally induce catabolic enzymes from the beginning. Monolayers were then washed with phosphate-buffered saline containing 2% bovine serum albumin in order to remove residual 1α,25-(OH)2D3 and then the medium was changed and the cells were incubated with 41.8 μg of OCT (10 μM) in 10 ml/dish Dulbecco’s minimal essential medium containing 1% bovine serum albumin for 0, 1, 3, 6, 12, and 24 h.

Lipid Extraction

Lipid extraction was performed according to the method of Bligh and Dyer (18) as modified by Makin et al. (19). The organic layer, containing unchanged OCT and lipid-soluble metabolites, was evaporated to dryness, and the residue redissolved in hexane/isopropanol/methanol (HIM), (91:7.2, v/v/v) for straight phase HPLC.

High Performance Liquid Chromatography

Straight phase HPLC was carried out using a model 590 pump, a U6K manual injector, and a model 990 photodiode array detector (all from Waters Associates, Milford, MA). This scanning spectrophotometer was used during metabolite purification to identify peaks possessing UV characteristics of the vitamin D cis-triene system (UVmax = 265 nm, UVmin = 228 nm, UVmaxmin = 1.75). Separations were achieved using Zorbax SIL (6.2 × 80 mm) or Zorbax CN (4.6 × 250 mm) columns (Dupont NEN). Mobile phases used for OCT metabolites included mixtures of HIM ranging from 91:7.2 to 94:5:1. Concentrations of stock solutions of OCT and its metabolites were assessed spectrophotometrically using a molar extinction coefficient, εmax = 18,300.

Purification of Metabolites

The lipid residue from the extraction of three 150-mm culture dishes containing HPK1A-ras (or HPK1A) cells was redissolved in 1.2 ml of HIM 91:7.2. Initial chromatography was performed on Zorbax SIL using HIM 91:7:2 at flow rate 1.0 ml/min. Six 200-μl aliquots of the extract were chromatographed, and peaks possessing the characteristic chromophore of vitamin D were collected and pooled. These fractions were subjected to further chromatography on Zorbax CN and Zorbax SIL. At this point, metabolites were pure enough for chemical derivatization and/or mass spectrometry.

Derivatization Reactions

Periodate oxidation and borohydride reduction of metabolites of OCT were carried out according to published methods (13, 20).

Gas Chromatography-Mass Spectrometry

Purified metabolites were derivatized to permethylsilyl ethers and then analyzed by GC-MS as described previously (20). Injection of metabolite mixture of OCT into the high temperature injection zone of the GC causes B ring closure producing pyro- and isopyroisomers. Mass spectra were obtained only from the pyroisomer, and, for simplicity, in discussion of fragmentation and in figures illustrating spectra obtained, the uncylized metabolite structure is used rather than that of the correct pyroisomer. Mass spectra were obtained by averaging each peak and subtracting the background.

Biological Properties of OCT and Its Metabolites

VDR Binding Assay—The binding affinities of OCT and its metabolites for the calf thymus vitamin D receptor were tested using a 1α,25-(OH)2D3 assay kit purchased from INCSTAR (Stillwater, MN). Calf thymus VDR was incubated at 20°C for 1 h with various concentrations of 1α,25-(OH)2D3 (1.25–80 pg/tube), OCT (1.25–80 pg/tube), or its metabolites (2.5 pg/tube to 205 ng/tube). After the incubation period, 15,000 dpm of [3H]1α,25-(OH)2D3 was added and incubated for 1 h at 20°C. Bound and free forms of [3H]1α,25-(OH)2D3 were separated by addition of a dextran/charcoal suspension and centrifugation. Radioactivity was measured with an Aloka LCS-90.

DBP Binding Assay—The binding affinities of OCT and its metabolites for the DBP were tested using plasma from a vitamin D-deficient rat. The displacement of [3H]1α,25-(OH)2D3 from 170,000 diluted vitamin D-deficient rat plasma in 3.5 ml barbiturate buffer (pH 8.6) containing 0.13 M NaCl and 0.1% ovalbumin was measured following the addition of 25-OH-D3 (12.5 pg/tube – 3.2 ng/tube), 1α,25-(OH)2D3 (1.6–820 ng/tube), OCT, or its metabolites (51 ng/tube to 52.4 μg/tube) as described previously (21). After incubation for 1 h at 4°C, bound and free forms of [3H]1α,25-(OH)2D3 were separated by the addition of a dextran/charcoal suspension and centrifugation. The supernatants containing bound [3H]1α,25-(OH)2D3 were transferred into scintillation vials, and radioactivity was measured in a scintillation counter.

Assessment of Cell Growth of HL-60 (22)—Human promyelocytic leukemic cells (HL-60 cells) were kindly provided by Dr. M. Inaba (Osaka University, Medical School). Cells were cultured at 37°C in RPMI 1640 medium (Nissui Pharmaceutical, Japan) supplemented with 10% heat-inactivated fetal calf serum and 60 μg/ml of kanamycin in a humidified atmosphere of 5% CO2 in air. Under these conditions the doubling time of HL-60 cells was 24 h. Vitamin D treatment involved seeding HL-60 cells at 1 × 104 cells/ml in growth medium and culturing for 72 h in the presence of from 10-10 M to 10-7 M of 1α,25-(OH)2D3, OCT, or its metabolites, each dissolved in ethanol. Control cultures were treated with ethanol vehicle at 0.1% (v/v). Cell viability was determined by trypan blue exclusion. The number of cells counted from triplicate experiments was expressed as a percentage of the control. The results are expressed as the mean of triplicate counts ± standard error.

RESULTS

Metabolism of OCT in the Human Keratinocyte Cell Line, HPK1A-ras

In an attempt to establish the full extent of metabolism of OCT, we began our studies by incubating this analog with the cell line HPK1A-ras, previously shown to have very high catabolic activity toward 1α,25-(OH)2D3 (15). When we incubated 10 μM OCT for 48 h, we obtained extensive metabolism such that on HPLC seven metabolite peaks possessing the vitamin D chromophore were discernible. These are labeled peaks 1–7 in Fig. 1, where peak 3 is the substrate, OCT, and the rest are metabolic products. Little substrate was left at the end of the 48-h period, whereas no metabolites were formed in no dead cell (microwave-treated) control plates incubated with
substrate OCT (and 5, and these metabolites remain unidentified at this time. We obtained limited information regarding peaks 1 and 5, and these metabolites remain unidentified at this time. Evidence that was obtained for the successful identification of OCT(Fig. 1A). All peaks 1–7 were purified by extensive rechromatography on Zorbax-CN and then again on Zorbax-SIL in order to identify them by GC-MS and chemical derivatization. In the case of peak 2, we made a sodium borodeuteride reduction product to identify them by GC-MS and chemical derivatization. In the case of peaks 4, 6, and 7, they were subjected to GC-MS as the permethylsilylated derivatives or, in the case of peaks 6 and 7, as their n-butyl boronate ester/permethylsilylated derivatives.

Identification of the Major Metabolites of OCT

We have summarized in Table I and detailed below the evidence that was obtained for the successful identification of the major metabolites. Peak 3 was identified as unchanged OCT(Fig. 1A). All peaks 1–7 were purified by extensive rechromatography on Zorbax-CN and then again on Zorbax-SIL in order to identify them by GC-MS and chemical derivatization. In the case of peak 2, we made a sodium borodeuteride reduction product to identify them by GC-MS and chemical derivatization. In the case of peaks 4, 6, and 7, they were subjected to GC-MS as the permethylsilylated derivatives or, in the case of peaks 6 and 7, as their n-butyl boronate ester/permethylsilylated derivatives.

Peaks showing the vitamin D chromophore are numbered 1–7. A shows the no cell control experiment. B shows the extract from HPK1A-ras cells. The HPLC conditions were Zorbax-SIL (3 μ; 6.2 × 80 mm); HIL 91:7:2, 1 ml/min.

Fig. 1. HPLC of extract of HPK1A-ras cells incubated with 10 μM OCT for 48 h. Peaks showing the vitamin D chromophore are numbered 1–7. A shows the no cell control experiment. B shows the extract from HPK1A-ras cells. The HPLC conditions were Zorbax-SIL (3 μ; 6.2 × 80 mm); HIL 91:7:2, 1 ml/min.

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Peak 2 (22,23,24,25,26,27-Hexanor-20-oxo-1α,20-dihydroxyvitamin D3)—Peak 2 was more strongly retarded than OCT at 14.91 min on Zorbax-SIL chromatography. Peak 2 was identified as unchanged OCT(Fig. 1A). All peaks 1–7 were purified by extensive rechromatography on Zorbax-CN and then again on Zorbax-SIL in order to identify them by GC-MS and chemical derivatization. In the case of peak 2, we made a sodium borodeuteride reduction product to identify them by GC-MS and chemical derivatization. In the case of peaks 4, 6, and 7, they were subjected to GC-MS as the permethylsilylated derivatives or, in the case of peaks 6 and 7, as their n-butyl boronate ester/permethylsilylated derivatives.

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conclude on the basis of extensive evidence that peak 6 is 24R-hydroxy-OCT.

Peak 7 ((25R)-26-Hydroxy-OCT)—This peak was the most polar metabolite obtained with a retention time of 23.99 min on Zorbax-SIL and 16.73 min on Zorbax-CN (Table I). Like peak 6, it proved to be sensitive to sodium metaperiodate, giving a product with a much reduced retention time (12.13 min versus OCT 11.35 min). On GC-MS, the pertrimethylsilylated derivative of peak 7 gave a molecular ion of \( m/z \) 722, suggesting a monohydroxylated derivative of OCT. The mass spectrum (Fig. 5A) featured many of the same ions found in the spectrum of peak 6 except that there was a large fragment at \( m/z \) 103 (corresponding to loss of \( \text{CH}_3\text{OTMS} \) instead of \( m/z \) 131 found in the case of 24-hydroxy-OCT. This fragment is indicative of a \( \text{CH}_3\text{OTMS} \) presumably vicinal to the existing C-25 hydroxy group at C-26 (C-27).

On GC-MS, the periodate cleavage product of peak 7 gave no discernible molecular ion, but the fragmentation pattern featured large fragments at \( m/z \) 456 (\( M^+ - 90 \)) and \( m/z \) 366 (\( M^+ - 90 \) – 90) and \( m/z \) 415 (\( M^+ - 131 \) due to loss of a fragment containing carbons C-2,3,4), data consistent with the molecular ion being extrapolated to be \( m/z \) 546 (Fig. 5C). This is consistent with a molecule truncated between C-25 and C-26 (C-27) and containing a C-25 ketone. Further confirmation of the peak 7 being a molecule with vicinal hydroxyls came from formation of an n-butylboronate derivative. As with peak 6 the permethylsilylated version of this was subjected to GC-MS and gave a molecular ion of \( m/z \) 644 (Fig. 5B). In addition to the distinctive fragment at \( m/z \) 169 found with the n-butyloboronate, permethylsilylated derivative of 24-hydroxy-OCT, we observed a distinctive fragment at \( m/z \) 141 due to cleavage of the C-24–C-25 bond and loss of a fragment containing the C-25, C-26, and C-27 butyloboronate ester group. Finally, peak 7 was shown to co-chromatograph with chemically synthesized (25R)-26-OH-OCT on HPLC. We conclude from these data that peak 7 must be (25R)-26-hydroxy-OCT.

### Table I: Summary of metabolites of OCT generated by human keratinocytes

| Peak | HPLC<sup>a</sup> | UV | Chemical derivatization<sup>b</sup> | Inferred molecular weight<sup>c</sup> | Putative structure |
|------|------------------|----|------------------------------------|-------------------------------------|-------------------|
|      | Zorbax SIL min  | Zorbax CN min | NaIO<sub>4</sub> | NaBD<sub>4</sub> |                      |
| 1    | 6.03             | 5.43 | 228 265                          | – +                                 | unknown           |
| 2    | 10.72            | 9.97 | 228 265                          | – –                                 | 330 22,23,24,25,26,27-hexanor-20-ketone |
| 4    | 14.91            | 10.87 | 228 265                          | – +                                 | 332 22,23,24,25,26,27-hexanor-20-OH |
| 5    | 16.32            | 15.89 | 228 265                          | – + (432)                            | unknown           |
| 6    | 17.16            | 13.17 | 228 265                          | + –                                 | 434 24-OH         |
| 7    | 23.99            | 16.73 | 228 265                          | + +                                 | 434 26-OH         |
| 3 (OCT) | 11.41         | 8.58 | 228 265                          | – –                                 | 418               |

<sup>a</sup> Zorbax SIL (6.2 × 80 mm), HIM 91:7:2, 1.0 ml/min; Zorbax CN (4.6 × 250 mm), HIM 91:7:2, 1.5 ml/min.

<sup>b</sup> – = no reaction; + = cleavage by periodate or reduction by borodeuteride.

<sup>c</sup> From TMS derivative run on GC-MS.
Comparison of Biological Products to Chemically Synthesized Standard Compounds

As a result of preliminary in vitro studies, both possible epimers of each biologically generated metabolite were synthesized chemically. Table II shows the results of HPLC co-migration studies of biological and chemically synthesized metabolites. In each case the epimeric mixture could be resolved on HPLC, and the biological product was found to be exclusively one epimer (that is peak 4 = 20S-epimer of the hexanor-1α,20- (OH)2D3, peak 6 = 24R-hydroxy OCT, and peak 7 = 25R-form of 26-hydroxy OCT). All chemically synthesized standard compounds were subjected to GC-MS for comparison to previously obtained mass spectra of biological products. Mass spectra (data not shown) were found to be virtually identical to those shown in Figs. 2–5 and therefore corroborate the identifications provided.

Time Course of OCT Metabolism in HPK1A-ras Cells

Evidence from the time course suggests that at early time points the hexanor-1,20-(OH)2D3 is present when there is no detectable hexanor-20-oxo-1α-OH-D3 (Fig. 6). All other metabolites accumulate at early time points, and their concentration plateaus or even decreases by the end of the incubation period (24 h).

Studies of the Metabolism of OCT in the HPK1A Keratinocyte, the Rat Osteosarcoma, UMR-106, and the Human Hepatoma, HepG2

In these studies we examined the metabolism of OCT using established cell lines involved in both the activation and catabolism of vitamin D. With such cell lines it was found that OCT was metabolized to a smaller number of products, which co-

TABLE II
Stereochemical configuration of peaks 4, 6, and 7 produced by HPK1A-ras cells

| OCT metabolite | Retention time on HPLC | System 1a | System 2b |
|----------------|------------------------|-----------|-----------|
| Peak 4         |                        | 15.87     | NDc       |
| Hexanor-1α,20S-(OH)2D3 (ED67) |          | 15.90     | NDc       |
| Hexanor-1α,20R-(OH)2D3 (ED190) |          | 14.15     | NDc       |
| Peak 6         |                        | 16.67     | 29.65     |
| 24R-OH-OCT     | (ED106)                | 18.70     | 29.33     |
| 24S-OH-OCT     | (ED107)                | 18.63     | 28.77     |
| Peak 7         |                        | 25.80     | NDc       |
| (25R)-26-OH-OCT (ED141) |          | 25.80     | NDc       |
| (25S)-26-OH-OCT (ED142) |          | 24.77     | NDc       |

a HPLC conditions: Zorbax-SIL, HIM 91:7:2, 1.0 ml/min.
b HPLC conditions: Zorbax-CN, HIM 91:7:2, 1.0 ml/min.
c ND, not determined.
d Chugai identification codes.
that OCT had virtually disappeared at the end of the 48-h period in HPK1A-ras cells, whereas considerable amounts remained in plates containing UMR-106 and HPK1A cells. The major metabolite found in cell cultures of UMR-106 and HPK1A was the 24-hydroxy-OCT (peak 6), whereas the truncated metabolites, peaks 4 and 2, appeared to be more important as the degree of metabolism increased (e.g. in HPK1A-ras). These data are consistent with the scheme shown in Fig. 9 with monohydroxylated versions of OCT acting as intermediates to the truncated metabolites, peaks 4 and 2.

**Biological Activities of OCT and Its Metabolites**

The VDR binding affinities of the 26-hydroxy-OCT isomers (ED141 and ED142) or side chain truncated metabolites of OCT (ED26, ED190, and ED67) were much reduced compared with the parent molecule, OCT, which showed approximately the same binding affinity for the mammalian VDR as 1α,25-(OH)2D3 (Fig. 10A). This was especially true of the side chain truncated metabolites of OCT, which showed a binding affinity 2–3 orders of magnitude lower than 1α,25-(OH)2D3. On the other hand, 24R-hydroxy-OCT (ED116) or 24-oxo-OCT (ED174) retained much of the VDR-binding affinity of OCT with values
The biological activity of the other epimer was determined, and the data corresponding to the biologically generated metabolite is illustrated. HL-60 cell growth. For clarity, only the chemically synthesized epimer between 1/2 and 1/7 that of 1 (ED106) or (25)-26-hydroxy-OCT (ED26) or the hexanor-20-oxo-compound (ED67) were used at a concentration between 1 x 10^-10 M and 1 x 10^-7 M. Significant inhibition of growth could be seen at a steroid concentration as low as 10^-9 M 1α,25-(OH)2D3, where the growth was decreased to <40% of control. The suppression of cell growth provided by OCT was virtually identical, whereas the suppressions caused by the hexanor-20-oxo-compound (ED26) or the hexanor-20S-hydroxy-compound (ED67) were greater than 2 orders of magnitude less effective than 1α,25-(OH)2D3. Other metabolites such as 24R-hydroxy-OCT (ED106) or (25R)-26-hydroxy-OCT (ED141) showed slightly inferior growth-inhibiting activity to OCT, with potencies between 1/2 and 1/3 that of 1α,25-(OH)2D3. The results of the biological activity studies for all chemically synthesized epimers, including biologically generated products of OCT, are summarized in Fig. 11.

DISCUSSION

We have described here the extensive metabolism of the vitamin D analog, 22-oxacalcitriol in a variety of cultured cell lines, including one hepatoma cell line and three vitamin D-target cell lines previously shown to catabolize 1α,25-(OH)2D3. Our study is the first to rigorously identify any of the metabolic products of OCT formed in vitro, and this was made possible by the generation of microgram quantities of these metabolites. The novel metabolites identified include two truncated versions of the OCT molecule lacking carbons 23-27 and containing a 20-oxo or 20-hydroxyl group. The 20-hydroxy compound has been reported before in vitro, but identification was based solely upon comigration with a chemically synthesized standard on HPLC (11). Here we were able to augment the preliminary identification afforded by HPLC-chromatography with GC-MS analysis and later comparison with chemically synthesized standards. Furthermore, our work is also consistent with the preliminary finding of a glucuronide conjugate of the 20-hydroxy compound in rat bile following OCT administration (10). The 24R-OH-OCT and 26-OH-OCT in the 25R-form identified here are novel, and their stereochemistry is also established.

The finding of both intact and truncated versions of OCT in cell extracts evokes the obvious question of how is OCT cleaved? Our venture into the metabolic fate of an oxygen containing molecule was not without precedent. In a previous study, we examined the metabolic fate of 24-oxa-1α-OH-D3 in the same hepatoma cell line HepG2 (23). In that case, we also found cleaved molecules, although the ultimate products were 23-substituted and lacked the carbon atoms C-25, C-26, and C-27 of the starting material. Thus, in the cases of both 22-oxacalcitriol and 24-oxa-1α-hydroxyvitamin D3, the molecule is cleaved just distal to the oxygen atom. In the case of 24-oxa-1α-OH-D3, we hypothesized that the enzymatic hydroxylation of a carbon vicinal to the oxygen atom would give rise to an unstable hemiacetal linkage that would undergo spontaneous breakdown to the C-23 substituted products (23). If we extrapolate from this result to the case of OCT we would predict that C-23 hydroxylation would have to occur in order to generate an unstable hemiacetal and truncated products retaining the 22-oxa group. The metabolites that were observed, namely 24-OH-OCT and 26-OH-OCT, would appear to be either precursors to this 23-hydroxylated intermediate or are by-products of the metabolic machinery. It is interesting to note that a 24-hydroxylated version of 1α,25-(OH)2D3 precedes a 23-hydroxylated metabolite in the catabolic sequence for the natural hormone during its conversion to calcitroic acid observed in a variety of target cell systems (19, 24). Alternatively, others have identified 26-hydroxylated derivatives of 25-OH-D3 (25) and 1α,25-(OH)2D3, and a 26-hydroxylated metabolite is observed as an intermediate during formation of the 26,23-lactone, but this is postulated to occur after and not prior to 23-hydroxylation (27). It remains to be proven if either 24-OH-OCT or 26-OH-OCT is a precursor to the 20-hydroxy and 20-oxo derivatives also found here.

Only in the case of the keratinocyte cell lines (HPK1A-ras and HPK1A) did we find appreciable quantities of the 20-oxo-truncated derivative of OCT. It is possible that the pattern of OCT metabolism that we observed, including the formation of the 20-oxo derivative, resulted from the induction of the catabolic enzymes caused by the high substrate concentrations that we employed. Interestingly, we observed the same pattern of metabolites including the 20-hydroxy and 20-oxo-truncated derivatives when [2β,3H]OCT (12 Ci/mmol) was incubated at low

![Fig. 10. Biological activity of the metabolites of OCT (relative to 1α, 25-(OH)2D3). A, VDR binding. B, DBP binding. C, inhibition of HL-60 cell growth. For clarity, only the chemically synthesized epimer corresponding to the biologically generated metabolite is illustrated. The biological activity of the other epimer was determined, and the data are depicted numerically in Fig. 11. ○, 1α,25-(OH)2D3; ●, OCT; □, 24R-OH-OCT; ▲, (25R)-26-OH-OCT; △, hexanor-20-oxo-1α-OH-D3; ▽, hexanor-1α,20S-(OH)2D3.](image-url)
nanomolar concentrations with HPK1A-ras cells (data not shown). The formation of the 20-oxo derivative could also be due to the different redox state of these cells compared with the other cell lines used. On the other hand, this may provide evidence of the role of one compound as an intermediate in the formation of the other (see Fig. 9). The time course study carried out suggests that the 20-hydroxy compound predates the 20-oxo derivative and thus may be converted to the latter in this culture system.

The biological activities of the metabolites identified here (where possible together with their epimeric forms; see Fig. 11) provides evidence that the in vivo properties of OCT can probably be ascribed to the parent compound and not to its principal metabolites. In all cases, metabolites of OCT were inferior to OCT (and 1α,25-(OH)₂D₃) in all parameters measured (i.e. VDR binding, DBP binding, and growth suppression of HL-60). This was particularly marked for the 20-oxo and 20-hydroxy derivatives where truncation of the side chain of OCT lowered biological activity by more than 2 orders of magnitude. A rough correlation between the strength of calf thymus VDR binding and the ability of the vitamin D analog to inhibit growth of HL-60 cells was still discernible. As with other 22-oxa analogs studied in this field (28), the calcium-transporting activity of the metabolites reported here remains unknown.

In the work described here, we observed OCT metabolism in both vitamin D target cells, namely, bone and keratinocyte, as well as a tissue involved in the activation of vitamin D, namely liver. It has been previously shown that like 1α,25-(OH)₂D₃ metabolism (19), OCT metabolism is vitamin D-inducible in cultured bovine parathyroid cells (11) and normal human peripheral monocytes (29). The result that hepatoma cells are also able to form truncated OCT metabolites is interesting given that liver cells are thought to be devoid of the vitamin D receptor and the vitamin D-inducible catabolic enzymes (e.g. cytochrome P-450 CYP24) (30, 31). However, small modifications of the vitamin D side chain have been shown to result in much more rapid hepatic metabolism and indeed 24-hydroxylation and 24-oxidation of certain analogs (e.g. calcipotriol) (32, 12, 15). Thus, it is possible that in addition to being susceptible to attack by vitamin D-inducible enzymes in classical vitamin D target cells, OCT is also attacked by more general enzymes in the liver leading to cleavage of the side chain by similar or slightly different mechanisms. It is clear from the studies presented here that OCT is more efficiently degraded by vitamin D-target cells than by hepatoma cells, and this may be important to the in vivo distribution and biological activity of the drug (33) as well as to the endogenous ligand (34).

Lastly, our finding of truncated versions of OCT would seem to open the door to study the origin of the conjugates found in bile in vivo following OCT administration to rats (10). The free 20-oxo or 20-hydroxy compounds identified here would likely be transported to the liver prior to conjugation to glucuronic acid in the hepatocyte, but this remains to be proven.

REFERENCES
1. Suda, T., Shinkii, T., and Takahashi, N. (1990) Annu. Rev. Nutr. 10, 195-211
2. Calverley, M. J., and Jones, G. (1992) in Antitumour Steroids (Blickenstaff, R. T., ed) pp 193-270, Academic Press, New York
3. Jones, G., and Calverley, M. J. (1993) Trends Endocrinol. Metab. 3, 297-303
4. Okano, T., Tsugawa, N., Masuda, S., Takeuchi, A., Kobayashi, T., and Nishii, Y. (1989) J. Nutr. Sci. Vitaminol. 35, 523-533
5. Abe, J., Morikawa, M., Miyamoto, K., Kaiho, S., Fukushima, M., Miya, A., Abe, E., Suda, T., and Nishii, Y. (1987) FEBS Lett. 226, 58-62
6. Abe, J., Takita, Y., Nakano, T., Miya, A., Suda, T., and Nishii, Y. (1989) Endocrinology 124, 2645-2647
7. Murayama, E., Miyamoto, K., Kuboda, N., Mori, T., and Matsunaga, I. (1986) Chem. & Pharm. Bull. (Tokyo) 34, 4410-4413
8. Takizawa, M., Fallen, M., Stein, B., and Epstein, S. (1992) Calciu. Tissue Int. 50, 521-523
9. Kobayashi, T., Okano, T., Tsugawa, N., Masuda, S., Takeuchi, A., and Nishii, Y. (1991) Contrib. Nephrol. 91, 129-133
10. Kobayashi, T., Tsugawa, N., Okano, T., Masuda, S., Takeuchi, A., Kuboda, N., and Nishii, Y. (1994) J. Biochem. 115, 373-380
11. Brown, A. J., Berkoben, M., Ritter, C., Kuboda, N., Nishii, Y., and Slatopolus, E. (1999) Biochem. Biophys. Res. Commun. 189, 759-764
12. Strugnell, S., Calverley, M. J., and Jones, G. (1990) Biochem. Pharmacol. 40, 333-341
13. Lohnes, D., and Jones, G. (1987) J. Biol. Chem. 262, 14394-14400
14. Henderson, J., Sebag, M., Rhim, J., Goltzman, D., and Kremer, R. (1991) Cancer Res. 51, 6521-6526
15. Masuda, S., Strugnell, S., Calverley, M., Makin, H. L. J., Kremer, R., and Jones, G. (1994) J. Biol. Chem. 269, 4794-4803

Fig. 11. Structures and biological activity data for chemically synthesized metabolites of OCT. The data are summarized for both stereochemical versions of each metabolite.
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16. Kubodera, N., Watanabe, H., Hatakeyama, S., Tazumi, K., and Takano, S. (1994) Bioorg. & Med. Chem. Lett. 4, 753–756
17. Sebag, M., Henderson, J., Rhim, J., and Kremer, R. (1992) J. Biol. Chem. 267, 12162–12167
18. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
19. Makin, G., Lohnes, D., Byford, V., Ray, R., and Jones, G. (1989) Biochem. J. 262, 173–180
20. Qaw, F., Calverley, M. J., Schroeder, N. J., Trafford, D. J. H., Makin, H. L. J., and Jones, G. (1993) J. Biol. Chem. 268, 282–292
21. Arnaud, S. B., and Meje, J. (1983) in Assay of Calcium Regulating Hormones (Bikle, D., ed) pp. 65–83, Springer-Verlag New York Inc., New York
22. Miyaura, C., Abe, E., Kuriyama, T., Tanaka, H., Konno, K., Nishi, Y., and Suda, T. (1981) Biochem. Biophys. Res. Commun. 102, 937–943
23. Calverley, M. J., Strugnell, S., and Jones, G. (1993) Tetrahedron 49, 739–746
24. Reddy, G. S., and Tserg, K. (1989) Biochemistry 28, 1763–1769
25. Hollis, B. W., Ross, B. A., and Lambert, P. W. (1980) Biochem. Biophys. Res. Commun. 95, 520–528
26. Reinhardt, T. A., Napoli, J. L., Pramink, B., Littledike, E. T., Beitz, D. C., Partridge, J. J., Uuskokovic, M. R., and Horst, R. L. (1981) Biochemistry 21, 6230–6235
27. Yamada, S., Nakayama, K., Takayama, H., Shinki, T., Takasaki, Y., and Suda, T. (1984) J. Biol. Chem. 259, 884–889
28. Kubodera, N., Watanabe, H., Miyamoto, K., Matsumoto, M., Matsuoka, S., and Kawanishi, T. (1993) Chem. Pharm. Bull. 41, 1659–1663
29. Kamamura, S., Galliomi, M., Kubodera, N., Nishi, Y., Brown, A. J., Slatopolsky, E., and Dusso, A. (1993) Endocrinology 123, 2719–2723
30. Lohnes, D., and Jones, G. (1992) J. Nutri. Sci. Vitaminol. Special Issue, 75–78
31. Jones, G. (1994) in The Second International Forum on Calcified Tissue and Bone Metabolism: Vitamin D and its Analogues, pp. 30–33, Chugai Pharmaceutical Co. Ltd., Tokyo, Japan
32. Sorensen, H., Binderup, L., Calverley, M. J., Hoffmeyer, L., and Rastrup-Andersen, N. (1990) Biochem. Pharmacol. 39, 391–393
33. Dusso, A. S., Negrea, L., Gunawardhana, S., Lopez-Hiliker, S., Finch, J., Mori, T., Nishi, Y., Slatopolsky, E., and Brown, A. J. (1991) Endocrinology 128, 1687–1692
34. Dusso, A. S., Negrea, L., Finch, J., Kamamura, S., Lopez-Hiliker, S., Mori, T., Nishi, Y., Brown, A. J., and Slatopolsky, E. (1992) Endocrinology 130, 3129–3134