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Contribution of Several Metabolites of the Vitamin D Analog 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25-$(OH)_2$ vitamin D_3 (KH 1060) to the Overall Biological Activity of KH1060 by a Shared Mechanism of Action

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ABSTRACT. The synthetic 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) analog 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25-$(OH)_2$vitamin D_3 (KH1060) is considerably more potent than its cognate hormone. The mechanism of action of KH1060 includes interaction with the vitamin D receptor (VDR). We previously showed that KH1060 increases VDR stability in ROS 17/2.8 osteoblastic cells by inducing a specific conformational change in the VDR. KH1060 is metabolized, both *in vivo* and *in vitro*, into several stable products. In the present study, we investigated whether these metabolites might contribute to the increased biological activity of KH1060. We found that the potencies of two of these metabolites, 24a-OH-KH1060 and 26-OH-KH1060, were similar to that of 1,25-$(OH)_2D_3$ in inducing osteocalcin production by the osteoblast cell line ROS 17/2.8. This report further showed that these metabolites had the same effects as KH1060 on VDR: they increased VDR stability in ROS 17/2.8 cells, while limited proteolytic analysis revealed that they caused a conformational change in the VDR, resulting in an increased resistance against proteolytic cleavage. Furthermore, as shown in gel mobility shift assays, both compounds clearly induced VDR binding to vitamin D response elements. Together, these results show that the potent *in vitro* activity of KH1060 is not only directed by the effects on the VDR conformation/stabilization of the analog itself, but also by certain of its long-lived metabolites, and emphasizes the importance of detailed knowledge of the metabolism of synthetic hormonal analogs. BIOCHEM PHARMACOL **59**;6:621–627, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. vitamin D receptor; vitamin D analogs; KH1060; metabolites; receptor conformation; receptor stability

The clinical usefulness of 1,25-$(OH)_2D_3$¶ in the treatment of cancer and immunological disorders is limited by its calcemic activity [1]. In an attempt to obtain agents with a more favorable therapeutic profile, numerous 1,25-$(OH)_2D_3$ analogs have been developed [2]. Some of these analogs exert increased *in vivo* and *in vitro* activity compared to 1,25-$(OH)_2D_3$. One of the most potent, KH1060, has very strong effects on *in vitro* cell growth and differentiation and has high immunosuppressive activity [3–6]. The mechanism(s) underlying the increased potency of KH1060 are not completely clear. Interaction with the VDR is crucial for the action of KH1060 [6]. In a previous study, we showed that, compared to 1,25-$(OH)_2D_3$, KH1060 potently increased VDR stability in ROS 17/2.8 osteoblastic cells. KH1060 also induced a different conformation of the VDR, resulting in an increased protease resistance which is in line with the VDR stability data [7]. Besides these VDR-localized mechanisms, the metabolic characteristics of the analogs might also be important. *In vitro* KH1060 is metabolized into at least 22 different compounds, including several stable and biologically active ones [8]. The formation of these metabolites might contribute to the increased biological activity of KH1060. In the present study, the 4 most abundant metabolites (24a-OH-KH1060, 24-OH-KH1060, 26a-OH-KH1060, and 26-OH-KH1060) were examined as to their capability to stimulate osteocalcin

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¶ *Abbreviations:* 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; KH1060, 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25-$(OH)_2$vitamin D_3; VDR, vitamin D receptor; VDRE, vitamin D response element; and α-MEM, alpha minimal essential medium.

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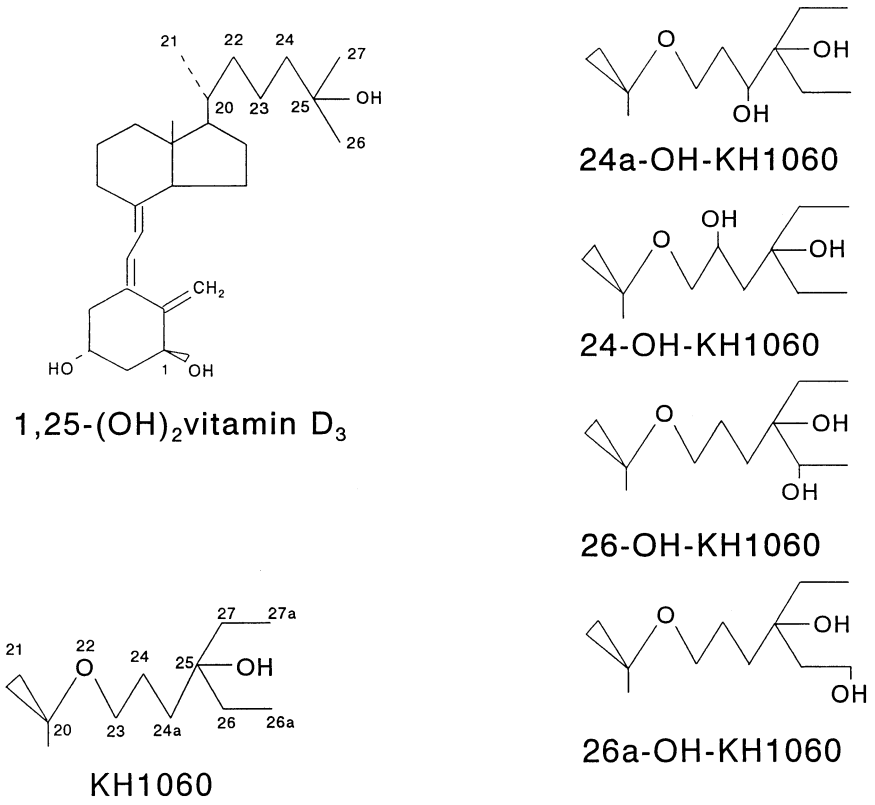

FIG. 1. Chemical structure of 1,25-$(OH)_2D_3$ and the modifications in the side chain of the synthetic analog KH1060 and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060.

synthesis in the osteoblast cell line ROS 17/2.8. Furthermore, the effects of these metabolites on VDR stability, VDR conformation, and VDR binding to VDREs were investigated.

MATERIALS AND METHODS

Materials

α-MEM and cycloheximide were from Sigma Chemical Co. L-Glutamine, penicillin, and streptomycin were from GIBCO Life Technologies, Inc. Fetal bovine serum was purchased from BioWhittaker. ^{32}P-ATP and [^{35}S]methionine were from Amersham. Ribonuclease inhibitor recombinant RNasin and the rabbit reticulocyte lysate assay were from Promega. Trypsin was from Boehringer Mannheim. Poly[dl-dC] was purchased from Pharmacia.

Generation, Extraction, and Purification of Metabolites of KH1060

HPK1A-*ras* cells (a gift from R. Kremer, Royal Victoria Hospital, McGill University, Montreal, Canada) were incubated with KH1060 (provided by L. Binderup and A.M. Kissmeyer, Leo Pharmaceutical Products) to generate KH1060 metabolites as described earlier [8]. The most abundant metabolites, i.e. 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060, were further examined in this study. Their identities were confirmed by GC–MS and their chemical structures are depicted in Fig. 1.

Osteocalcin Production Measurements

The rat osteoblast-like cell line ROS 17/2.8 (provided by S.B. Rodan, Merck, Sharp & Dohme) was cultured for 24 hr with vehicle, or with 1,25-$(OH)_2D_3$, KH1060, or the metabolites (10^{-14}–10^{-8} M) as described earlier [6]. Osteocalcin production was measured by radioimmunoassay [9].

VDR Stability Study

As described earlier [7], the ROS 17/2.8 cells were seeded in 100 × 15-mm tissue culture dishes and cultured for 2 days in α-MEM supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.1% D-glucose, and 10% fetal bovine serum. At 80% confluency, the medium was changed to α-MEM containing 2% charcoal-treated fetal bovine serum and 10 μM cycloheximide to block translation and thereby new synthesis of VDR. Next, vehicle, 1,25-$(OH)_2D_3$, KH1060, or its metabolites (1 nM) were added and after 4 and 24 hr cell extracts were prepared [10]. In the extracts, VDR content was measured using an enzyme-linked immunoassay [11].

In Vitro *Transcription and Translation*

Human VDR cDNA [12], a gift from M. R. Haussler of the University of Arizona, was *in vitro* transcribed and translated in the presence of [^{35}S]methionine (specific activity 1000 Ci/mmol) and ribonuclease inhibitor recombinant RNasin, using a rabbit reticulocyte lysate assay according to the manufacturer's instructions.

Limited Proteolytic Digestion of In Vitro *Synthesized VDR*

In vitro synthesized VDR was incubated with 1,25-$(OH)_2D_3$, KH1060, or the metabolites (10^{-12}–10^{-9} M, 20 min, room temperature) and subsequently treated with trypsin (25 μg/mL) for 10 min at room temperature. Trypsin concentration-dependent (0–500 μg/mL) resistance was tested at 10 nM ligand. The labeled fragments were separated on a 12.5% (w/v) polyacrylamide gel and visualized by exposure to Fuji RX medical x-ray film.

Gel Mobility Shift Assay

Gel shift assays with nuclear extracts from ROS 17/2.8 cells treated for 1 hr with vehicle, 1,25-$(OH)_2D_3$, KH1060, or the metabolites (1 nM) were performed as described earlier [13]. The ^{32}P-Labeled rat osteocalcin (5′-CTGCACTGGGTGAATGAGGACATTACTGA-3′) and rat cytochrome P450 (CYP24) VDRE oligo (5′-CGCGAGGTGAGTGAGGGCGCCGC-3′) were incubated with 5 μg of nuclear protein in a final KCl concentration of 50 mM and in the presence of 0.1 μg/μL of poly[dI-dC] nonspecific competitor DNA. The protein–DNA complexes were electrophoretically separated on a 5% non-denaturing polyacrylamide gel in 0.5 × TBE (0.045 M Tris-borate; 0.001 M EDTA) buffer and visualized by autoradiography. The shifted probe was scanned from the autoradiograph, and optical densities were expressed relative to the optical density of the shifted probe after vehicle treatment. For reasons of clarity, standard deviations (always smaller than 10%) were not depicted in most of the figures.

RESULTS

Effect of 1,25-$(OH)_2D_3$, KH1060, and the Metabolites on Osteocalcin Production by ROS 17/2.8 Cells

Figure 2 shows that 1,25-$(OH)_2D_3$, KH1060, and the KH1060 metabolites induced osteocalcin production in a dose-dependent manner. On the basis of EC_{50}, KH1060 was the most potent analog (4.5×10^{-13} M) followed by 24a-OH-KH1060 (1.3×10^{-11} M), 1,25-$(OH)_2D_3$ (7.2×10^{-11} M), 26a-OH-KH1060 (1.2×10^{-10} M), 26-OH-KH1060 (2.5×10^{-10} M), and 24-OH-KH1060 (1.4×10^{-9} M). All metabolites and 1,25-$(OH)_2D_3$ induced osteocalcin production with a similar maximum, whereas KH1060 had a somewhat lower maximal response.

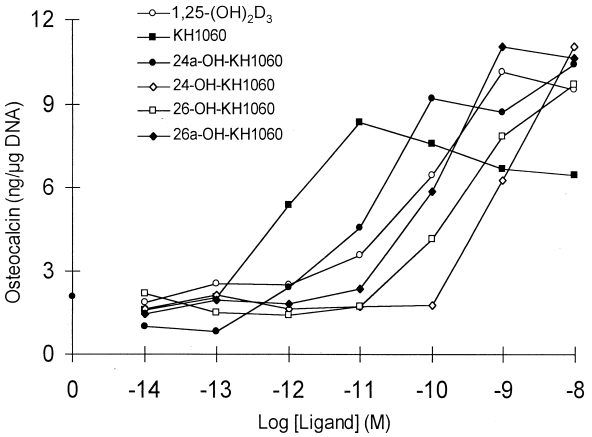

FIG. 2. Effect of 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 on the synthesis of osteocalcin by ROS 17/2.8 cells. Cells were treated for 24 hr with vehicle or with 1,25-$(OH)_2D_3$, KH1060 or its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 (10^{-14}–10^{-8} M), and osteocalcin secreted into the medium was determined as described in Materials and Methods. Each point represents the mean of two independent cultures in duplicate.

VDR Half-Life in ROS 17/2.8 Cells

Figure 3 shows the ligand-induced stabilization of the VDR in ROS 17/2.8 cells. In the absence of ligand, VDR was rapidly degraded. At 1 nM, 1,25-$(OH)_2D_3$, KH1060, and its metabolites increased the VDR half-life in ROS 17/2.8 cells, although, there was a marked difference in potency. Incubation with KH1060 and 24a-OH-KH1060 resulted in the most potent stabilization of the VDR. After 24 hr incubation, still about 60% (KH1060) and 45% (24a-OH-KH1060) of the initial VDR content was present. The other metabolites stabilized the VDR comparably to 1,25-$(OH)_2D_3$, while only 24-OH-KH1060 seemed less effective.

Limited Proteolytic Digestion of In Vitro *Synthesized VDR*

As shown in Fig. 4, A and B, 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 all protected, in a dose-dependent manner, *in vitro* synthesized VDR against trypsin activity. There was a conservation of distinct fragments of 32, 30, and 27 kDa (Fig. 4, A and C). We found that VDR incubated with KH1060 or its metabolites 24a-OH-KH1060 and 26-OH-KH1060 was less sensitive to trypsin than VDR incubated with 1,25-$(OH)_2D_3$ or 26a-OH-KH1060. The metabolite 24-OH-KH1060 was virtually ineffective in protecting VDR against protease action. The effects on VDR conformation were further studied by taking a fixed ligand concentration (10 nM) and a dose range of trypsin (Fig. 4, C and D). In addition, in this set-up, the same order of potency of the ligands to protect the 32-kDa product was observed.

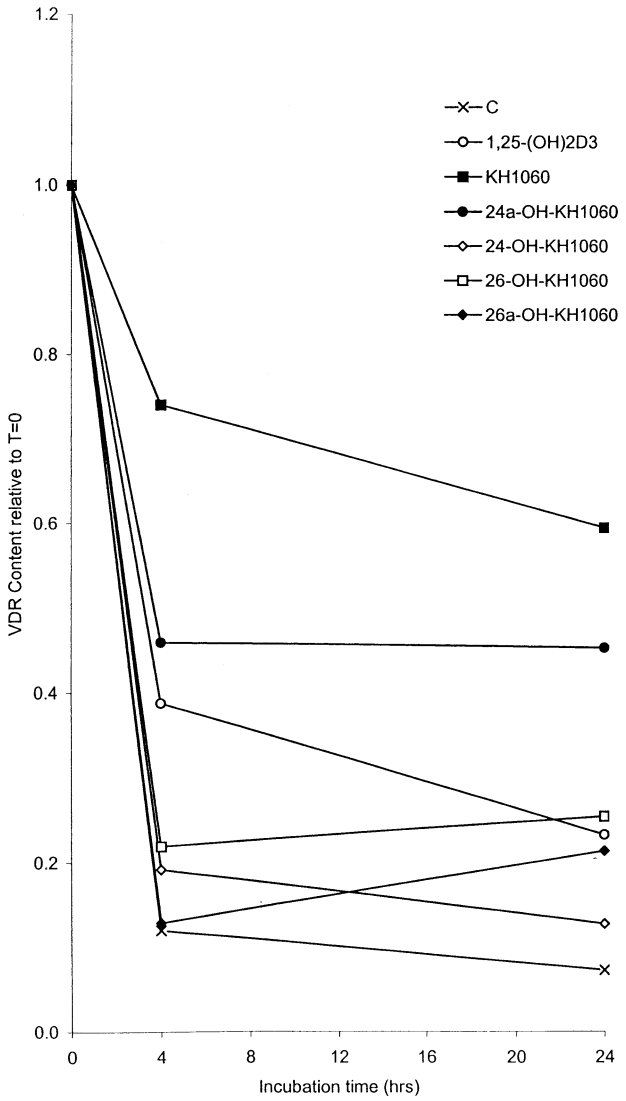

FIG. 3. Effect of 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 on VDR half-life in ROS 17/2.8 cells. Cycloheximide-treated ROS 17/2.8 cells were incubated for 4 or 24 hr with vehicle or with 1,25-$(OH)_2D_3$, KH1060 or its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 (1 nM). Then, extracts were prepared and assayed for VDR content by enzyme-linked immunoassay. Data represent the means of three independent experiments and were expressed as VDR content relative to T = 0.

Gel Mobility Shift Assays

In order to assess whether the KH1060 metabolites induce binding of the VDR to DNA, gel shift analyses were performed. As shown in Fig. 5, 1,25-$(OH)_2D_3$, KH1060, and its metabolites induced binding of the VDR to the rat osteocalcin VDRE, with KH1060 and 24a-OH-KH1060 the most active. 26-OH-KH1060 and 26a-OH-KH1060 were also more active than 1,25-$(OH)_2D_3$ in stimulating VDR binding to the osteocalcin VDRE, while 24-OH-KH1060 was least effective. With the CYP24 VDRE, KH1060 metabolite-induced VDR binding was also observed, and again 24-OH-KH1060 was least effective (data not shown). The shifted band (marked by the arrowhead) could be disrupted by adding the anti-VDR monoclonal antibody IVG8C11 (11) (data not shown).

DISCUSSION

The present study addresses the important issue of the metabolism of synthetic hormone analogs and the biological activity and mechanism of action of the metabolites formed. Previously, we showed that part of the increased biological potency of the 1,25-$(OH)_2D_3$ analog KH1060 probably lies in a specific interaction with the VDR. KH1060 enhanced the VDR half-life in ROS 17/2.8 cells [7] and induced an altered conformational change in the VDR [7, 14, 15]. Besides these VDR-based mechanisms, metabolic aspects (e.g. increased metabolic stability or formation of biologically active metabolites) might also contribute to the increased potency of KH1060. For other potent analogs of 1,25-$(OH)_2D_3$, such as 1(*S*),3(*R*)-dihydroxy-20(*R*)-5′-ethyl-5′-hydroxy-hepta-1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(Z),7(*E*),10(19)-triene (EB1089), 20-epi-1,25-dihydroxyvitamin D_3 (MC1288), and 26,26,26,27,27,27-hexafluoro-1,25-$(OH)_2D_3$, *in vivo* and *in vitro* metabolic stability could play a role in their increased potency [16–19]. However, for KH1060, the stability of the compound itself is not likely to be an important factor, since both *in vivo* and *in vitro* KH1060 is metabolized at a rate comparable to or faster than that of 1,25-$(OH)_2D_3$ [8, 18].

In addition to the metabolic stability of the analogs, the metabolites generated can also contribute to the biological potency of an analog. Both biological activity and stability determine the impact of metabolites on the eventual biological potency of a parent compound. The biological activity of a metabolite does not necessarily have to surpass the activity of the parent compound to contribute to the overall effect. A metabolite with modest activity but increased stability will also add to the eventual effect [20, 21]. An example of generation of metabolites with increased stability and significant biological activity is 26,26,26,27,27,27-hexafluoro-1,23,25-$(OH)_3$vitamin D_3, the major metabolite of 26,26,26,27,27,27-hexafluoro-1,25-$(OH)_2D_3$, which demonstrated distinct transcriptional activity in a reporter gene expression system [22]. Another example is the target tissue-specific 3β-hydroxy epimerization of 1,25-$(OH)_2D_3$ [23] and (potentially) its analogs. However, for the metabolites of the 1,25-$(OH)_2D_3$ analogs EB1089 [19, 24], 22-oxa-1,25-dihydroxyvitamin D_3 (OCT) [25], and 1,24(*S*)-dihydroxy-22-ene-25,26,27-cyclopropylvitamin D_3 (MC903) [26], no contribution of the metabolites to the biological potency of the parent compound could be assessed.

The supposition that metabolites of KH1060 might also add to the biological potency of the parent compound is underscored by the finding that several of its *in vitro*

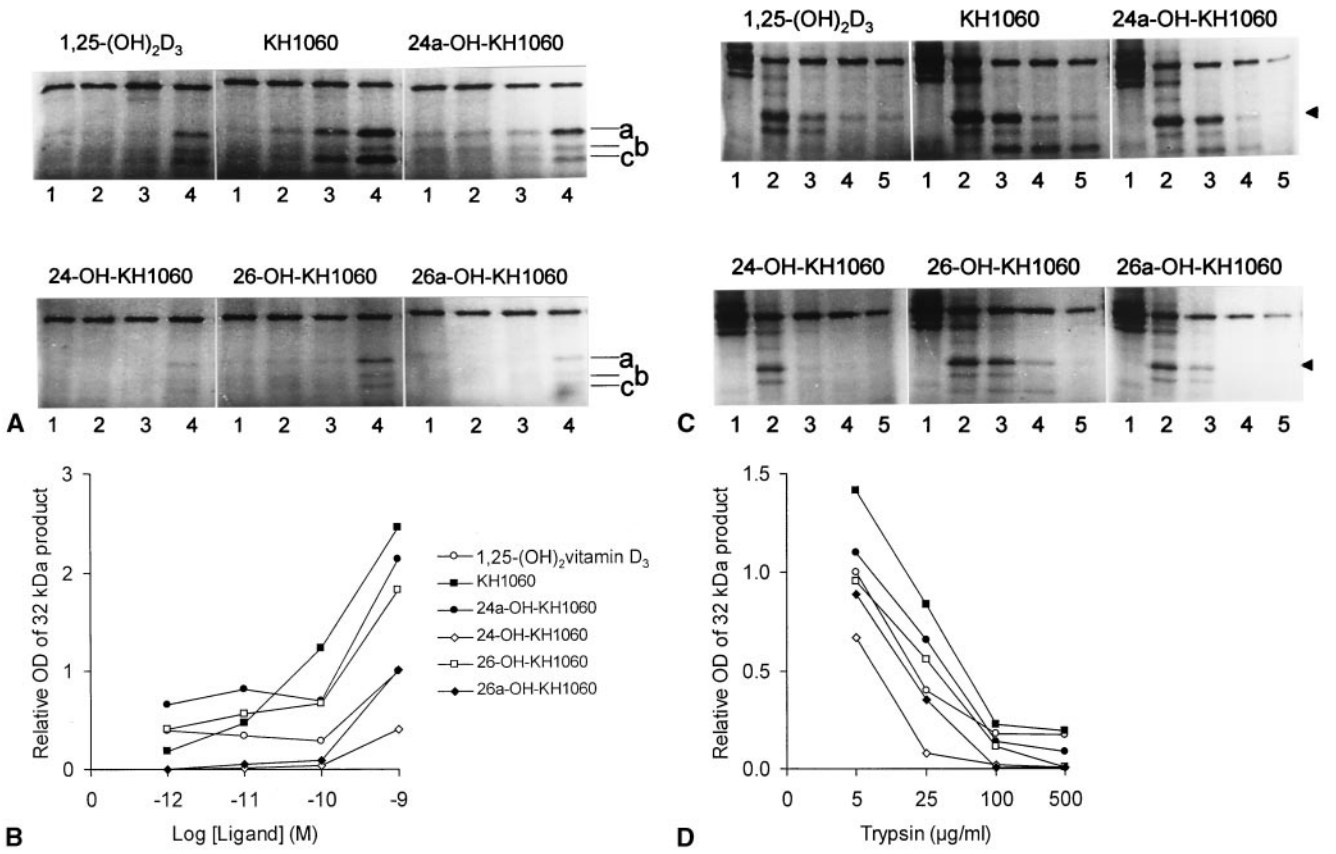

FIG. 4. Effect of 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 on VDR conformation. In panel A, *in vitro* synthesized human VDR was incubated with increasing concentrations of 1,25-$(OH)_2D_3$, KH1060, or its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060. Then, VDR protein was treated with trypsin (25 µg/mL), and the protease-resistant fragments (32, 30, and 27 kDa, marked a, b and c, respectively) were analyzed by SDS/PAGE. The ligand concentrations tested, 10^{-12}–10^{-9} M, are indicated by 1–4. In panel B, a computerized optical density scan (mean of two experiments) of the 32 kDa fragment (marked 'a' in panel A) at increasing ligand concentrations is shown. The O.D.s of the 32-kDa product were normalized to the effect of 1 nM 1,25-$(OH)_2D_3$. A representative gel of a trypsin concentration-dependent degradation of *in vitro* synthesized VDR treated with 10 nM 1,25-$(OH)_2D_3$, 10 nM KH1060, or 10 nM of its metabolites is shown in panel C. The trypsin concentrations tested, 0-5-25-100-500 µg/mL, are indicated by 1–5. Panel D represents a computerized optical density scan (mean of two experiments) of the 32-kDa product (marked by the arrowheads in panel C) normalized to the 1,25-$(OH)_2D_3$ effect at 5 µg/mL trypsin. For legend, see panel B.

generated metabolites (e.g. 24a-OH-KH1060 and 26-OH-KH1060) retained *trans*-activating and mRNA-inducing activity [8]. In addition, the metabolites of KH1060 might contribute to the biological activity of the analog *in vivo*: in serum and in liver tissue taken from rats injected with KH1060, both 24a-OH-KH1060 and 26-OH-KH1060 could be identified [8], and 24a-OH-KH1060 was detected in pig liver incubations [27]. Here, we extend the observation that KH1060 metabolites are biologically active and elucidate mechanisms involved in their action. Some of the metabolites were as potent as 1,25-$(OH)_2D_3$, while others were somewhat less potent but still able to induce osteocalcin production by ROS 17/2.8 cells. An interesting aspect of these metabolites is that they are stable. Even after 72 hr, they could be detected in cells treated with KH1060 [8]. This is in marked contrast to the metabolites of 1,25-$(OH)_2D_3$, which disappear very rapidly [28].

Analysis of the possible mechanism(s) involved in the action of these metabolites demonstrated that, like the parent compound [7], they affect VDR stability. The metabolites 24a-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 enhance VDR stability comparably to or more than 1,25-$(OH)_2D_3$. The metabolites 24a-OH-KH1060 and 26-OH-KH1060 also induce conformational changes in the VDR that result in an increased resistance to proteolytic cleavage compared to 1,25-$(OH)_2D_3$, while 26a-OH-KH1060 exerts similar effects as 1,25-$(OH)_2D_3$. Gel shift analysis with nuclear extracts from ROS 17/2.8 cells revealed that KH1060 metabolites also induce binding of the VDR to VDREs of vitamin D responsive genes. The low activity of 24-OH-KH1060 on the biological processes presented here underscores the significance of hydroxylation at C-24 in the 1,25-$(OH)_2D_3$ side chain in the inactivation of the hormone. In contrast, hydroxylation at C-24a only leads to marginal reduction in biological activity.

From a conceptual point of view the present data are interesting. Not only the parent molecule itself but also its

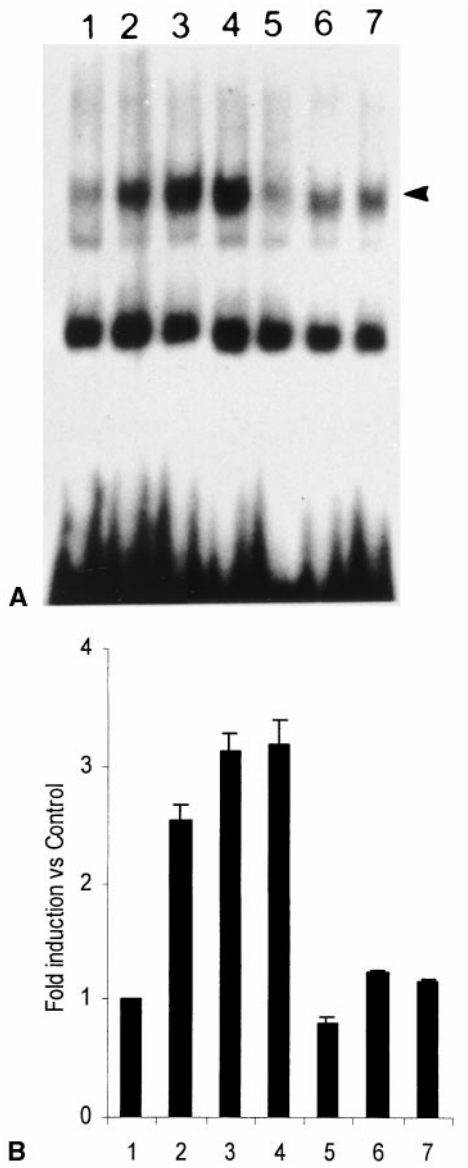

FIG. 5. Gel mobility shift assay with the rat osteocalcin VDRE probe and nuclear extracts of ROS 17/2.8 cells after 1-hr incubation with vehicle (lane 1), 1 nM 1,25-$(OH)_2D_3$ (lane 2), 1 nM KH1060 (lane 3) or 1 nM of its metabolites 24a-OH-KH1060 (lane 4), 24-OH-KH1060 (lane 5), 26-OH-KH1060 (lane 6), and 26a-OH-KH1060 (lane 7) is presented in panel A. A computerized optical density scan (mean ± SD of two experiments) of the shifted probe is presented in panel B. The optical density of the shifted probe after vehicle treatment was set to 1.

(long-lived) metabolites may exert effects and act via similar receptor-mediated mechanisms. In so doing, metabolites can significantly contribute to the eventual biological effect of an analog. Therefore, knowledge of both the metabolism of synthetic analogs and of the mechanism of action of the metabolites formed is of utmost importance. It is conceivable that this is not only applicable to vitamin D analogs, but also to other hormone analogs such as estrogen-like compounds, which have been the object of much recent attention [29–31]. An additional aspect in the concept of the metabolism of synthetic analogs is target tissue-specific metabolism. Not only may synthetic analogs be metabolized to different, more active metabolites than the natural occurring counterpart, but this metabolism may also be target cell/tissue-specific [23]. This latter aspect may also be part of the clinically interesting target tissue-specific effects of vitamin D analogs as well as estrogen antagonists.

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