25-Azavitamin D₃, an Inhibitor of Vitamin D Metabolism and Action*

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Bruce L. Onisko, Heinrich K. Schnoes, and Hector F. DeLuca
From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

25-Azavitamin D₃ inhibited both the bone calcium mobilization and intestinal calcium transport responses of rats to vitamin D₃ but not to 25-hydroxyvitamin D₃. Although 25-azavitamin D₃ had no effect on the response of bone to 1α,25-dihydroxyvitamin D₃, it did diminish the response of the intestine to that metabolite. 25-Azavitamin D₃ increased liver vitamin D content and reduced the concentration of 25-hydroxyvitamin D₃ in the serum. The doses of 25-azavitamin D₃ required to inhibit the metabolism of vitamin D₃ (75 and 200 μg) were similar to the doses of 25-azavitamin D₃ required to inhibit the action of vitamin D₃ in vivo (50 and 150 μg). 25-Azavitamin D₃ is thus a vitamin D antagonist, acting for the most part via inhibition of the liver 25-hydroxylation of vitamin D₃.

The hypercalcemia of a variety of disorders of calcium metabolism is the result of excessive intestinal transport of calcium or demineralization of bone (1, 2). Since both of these physiological processes require vitamin D (3, 4), an antagonist of vitamin D action should effectively lower the hypercalcemia. It is now known that the biological activity of vitamin D₃ in intestine and bone is the result of metabolic conversion to the hormone 1α,25-dihydroxyvitamin D₃ (5, 6). A consequence of the obligatory nature of the two-step metabolic activation of vitamin D is that inhibitors of vitamin D metabolism should block the expression of the biological responses to vitamin D₃. The metabolism of vitamin D₃ is sequential; it involves hydroxylation of vitamin D₃ at C-25 (primarily in the liver) to form 25-hydroxyvitamin D₃ and then hydroxylation of the latter at C-1 in the kidney to produce 1α,25-(OH)₂D₃ (7). Of the two alternatives, inhibition of the 25 hydroxylation is more desirable since it precludes the potential accumulation of 25-OH-D₃ which can directly give rise to responses in bone and intestine when present in a much higher than normal concentration (8, 9). Toward this end, we have prepared a variety of side chain modified analogs of vitamin D as potential 25-hydroxylase inhibitors (10). We wish to report that one of these compounds, 25-azavitamin D₃, does indeed antagonize the action of vitamin D₃ and that the mechanism of this antagonism is via inhibition of the 25-hydroxylation of vitamin D₃.

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The abbreviations used are: 1α,25-(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; 25-OH-D₃, 25-hydroxyvitamin D₃; 25-N-D₃, 25-azavitamin D₃.
fractions by evaporation, and the residues were redissolved in toluene counting solution. Tritium content was determined by use of a Packard Tri-Carb model 3575 liquid scintillation counter. Column recoveries were 76 ± 12% (mean ± S.D., n = 26).

Metabolite levels (Tables II, III, and IV) are expressed as a percentage of the total administered dose present in the organ examined. These values were readily obtained for liver samples, since the entire organ was extracted. The total serum volume of a rat (in milliliters) was approximated to be 3% of the total body weight in grams (17). This estimation and knowledge of the volume of serum extracted permitted calculation of the serum metabolite levels in the desired units.

### RESULTS

The effect of 25-N-D₃ on the biological activity of vitamin D₃, 25-OH-D₃, and 1α,25-(OH)₂D₃ was tested in the rat. Vitamin D-deficient animals, maintained on a low calcium diet, received graded doses of 25-N-D₃ by intragastric injection (Dose 1). Two hours after this dose, each rat was administered a physiological dose of either vitamin D₃ (50 ng), 25-OH-D₃ (25 ng), 1α,25-(OH)₂D₃ (10 ng), or the vehicle (ethanol) by the same route (Dose 2). Two physiological responses dependent upon vitamin D were assayed 20 h after the second dose. The mobilization of bone calcium was indirectly observed as a rise in serum calcium concentration. Intestinal calcium transport was measured using an everted duodenal segment. The results are summarized in Table I. Controls receiving only ethanol (Group 1) are adequately deficient in both vitamin D and calcium, as judged by their low values of serum calcium and intestinal transport. A dose of either vitamin D₃ (Group 2), 25-OH-D₃ (Group 5), or 1α,25-(OH)₂D₃ (Group 8) significantly elevated both parameters, as expected, whereas 25-N-D₃ (50 µg, Group 11) had no effect by itself. When given before the dose of vitamin D₃, 25 N-D₃ (50 µg, Group 3) significantly reduced both the bone and intestinal response to vitamin D₃ (p < 0.001). A larger dose of 25-N-D₃ (150 µg, Group 4) further reduced these responses to the control level of vitamin D-deficient animals (Group 1). 25-N-D₃ at either 50 µg (Group 6) or 150 µg (Group 7), did not significantly affect the bone or intestinal response of rats to 25-OH-D₃. 25-N-D₃ also had no effect on the bone response to 1α,25-(OH)₂D₃ (Groups 9 and 10). However, in the animals receiving the high dose of 25-N-D₃ (150 µg, Group 10), the intestinal transport to 1α,25-(OH)₂D₃ was significantly reduced.

We next examined the effect of 25-N-D₃ on the metabolism of vitamin D₃ to 25-OH-D₃ in the rat. Vitamin D-deficient animals received either solvent (EtOH) or 25-N-D₃ (200 µg/rat) by injection into the jugular vein. Two hours later, each animal received a physiological dose of [1,2 ³H]vitamin D₃ by the same route. At 4, 8, 12, 16, and 20 h after the radioactive dose, a pair of rats (±25-N-D₃) was killed; serum and liver samples were extracted and chromatographed on Sephadex LH-20; metabolite levels were quantitated by liquid scintillation counting. Fig. 1 shows an example of the Sephadex LH 20 chromatograms of the metabolites extracted from serum and liver samples obtained 12 h after the vitamin D₃ dose, whereas Tables II and III summarize the metabolite levels in the serum and liver of rats at all time points examined. The chromatographic profile of the serum and liver extracts from animals given radiolabeled vitamin D₃ (Fig. 1, top panel) show the presence of 25-OH-D₃ (Fractions 20 to 36), vitamin D₃ (Fractions 10 to 18), and esters of vitamin D₃ (Fractions 7 to 9) (17). (More polar metabolites elute in the column wash of

![Fig. 1. Sephadex LH-20 column chromatographic profiles of liver and intestinal extracts of rats given 25-N-D₃ and [³H]D₃. Vitamin D-deficient rats were dosed with either 25-N-D₃ (200 µg) or solvent (0.05 ml of EtOH) 2 h before an intrajugular dose of [1,2-³H]vitamin D₃. Twelve hours after the second dose, the animals were killed and their serum and liver were extracted and then the extracts were chromatographed as described under "Materials and Methods." Values have been appropriately adjusted to represent the extraction of equivalent volume of serum (1.0 ml).]

| Table I |
|---------|
| Response of intestine and bone to vitamin D and its metabolites in the presence of graded doses of 25-N-D₃ |
| Group | Dose 1 | Dose 2 | Intestinal calcium mobilization* ± S.E. | Bone calcium mobilization* ± S.E. |
|-------|--------|--------|--------------------------------|---------------------------------|
| 1 | Ethanol | Ethanol | 1.9 ± 0.1 (18) | 4.6 ± 0.1 (17) |
| 2 | Ethanol | D₃, 50 ng | 3.6 ± 0.1 (20) | 5.6 ± 0.1 (20) |
| 3 | 25-N-D₃, 50 µg | D₃, 50 ng | 2.8 ± 0.2 (16) | 5.0 ± 0.1 (16) |
| 4 | 25-N-D₃, 150 µg | D₃, 50 ng | 1.9 ± 0.1 (6) | 4.8 ± 0.1 (6) |
| 5 | Ethanol | 25-OH-D₃, 25 ng | 3.4 ± 0.2 (13) | 5.5 ± 0.2 (19) |
| 6 | 25-N-D₃, 50 µg | 25-OH-D₃, 25 ng | 3.1 ± 0.3 (6) | 5.4 ± 0.1 (6) |
| 7 | 25-N-D₃, 150 µg | 25-OH-D₃, 25 ng | 3.4 ± 0.3 (6) | 5.4 ± 0.1 (6) |
| 8 | Ethanol | 1α,25-(OH)₂D₃, 10 ng | 3.5 ± 0.1 (12) | 5.4 ± 0.1 (12) |
| 9 | 25-N-D₃, 50 µg | 1α,25-(OH)₂D₃, 10 ng | 2.9 ± 0.3 (6) | 5.2 ± 0.1 (6) |
| 10 | 25-N-D₃, 150 µg | 1α,25-(OH)₂D₃, 10 ng | 2.3 ± 0.2 (6) | 5.2 ± 0.1 (6) |
| 11 | Ethanol | 25-N-D₃, 50 µg | 1.5 ± 0.1 (4) | 4.4 ± 0.1 (4) |

* Serum Ca/mucosal Ca.

** Numbers in parentheses represent number of rats. (This table is the summary of three experiments, each of which had groups of four to seven animals. No significant differences between duplicate groups were noted and thus the appropriate data were combined.)

* Significant difference from control receiving same second dose but ethanol as the first dose at p < 0.001.

* No significant difference from control receiving same second dose but ethanol as the first dose (p > 0.05).
Data summarized in Table I show that doses of 50 or 150 μg of 25 N-D₃ effectively antagonize the bone and intestinal responses of vitamin D-deficient animals to vitamin D₃. Intestinal and bone responses to 25-OH-D₃ however, are not affected by treatment with 25-N-D₃. These observations, and knowledge of the essential metabolism of vitamin D₃ to 1α,25-(OH)₂D₃ via the intermediate 25 OH D₃, (7) imply that 25 N-D₃ acts by inhibition of the conversion of vitamin D₃ to 25-OH-D₃. The experiments examining the effect of 25-N-D₃ on the metabolism of [³H]vitamin D₃ show that this is indeed the case. The normal metabolism of vitamin D consists of rapid clearance of vitamin D₃ from the blood with simultaneous sequestration by the liver; hepatic vitamin D₃ content then declines, accounting for the production of 25-OH-D₃ (17). Pretreatment of vitamin D-deficient rats with 25-N-D₃ greatly retards this rise in serum 25-OH-D₃ content (Tables II and IV). The inhibition of the in vivo production of 25-OH-D₃ by 25-N-D₃ is not a result of impaired liver uptake of vitamin D₃ since liver vitamin D₃ content in the 25-N-D₃-treated rats is, in fact, found to be higher than in the ethanol-dosed controls. These two major disturbances of vitamin D metabolism (low serum 25-OH-D₃ and elevated liver vitamin D₃) could be a consequence of either inhibition of the enzymatic conversion of vitamin D₃ to 25-OH-D₃ by 25-N-D₃, or of inhibition of the transport of 25-OH-D₃ out of the liver. This latter possibility cannot be ruled out, but the slightly below normal liver 25-OH-D₃ levels found in 25 N-D₃-dosed animals suggest that direct inhibition of the hydroxylase is the more probable explanation.

In addition to the inhibition of the conversion of vitamin D₃ to 25-OH-D₃ by 25-N-D₃, 25-N-D₃ must have a secondary effect since administration of 25-N-D₃ and 1α,25-(OH)₂D₃ to rats results in an inhibition of the intestinal response to 1α,25-(OH)₂D₃ (Table I). This inhibition is not expected, given the inability of 25-N-D₃ to depress the calcium transport response to 25-OH-D₃, a metabolite which acts via metabolism to 1α,25-(OH)₂D₃. The apparent paradox may be the result of the different time course of response of 25-OH-D₃ and 1α,25-(OH)₂D₃ and thus it might be possible to demonstrate an inhibition of the intestinal response to 25-OH-D₃ by later and more frequent administration of 25-N-D₃. This rationalization is most speculative, and the explanation must await the results of further investigation. In any case, the inhibition of the intestinal response to 1α,25-(OH)₂D₃ by 25-N-D₃ is of considerable interest. It demonstrates direct antagonism toward the hormonal form of vitamin D-1α,25-(OH)₂D₃. Furthermore, this antagonism is selective since no effect on the bone response to 1α,25-(OH)₂D₃ is observed. 25-N-D₃ is the first known vitamin D analog to possess either of these properties.

Other agents are known to antagonize the expression of vitamin D activity. Glucocorticoids have been shown to inhibit the intestinal calcium transport response to vitamin D₃ (18), 25-OH-D₃ (19), and 1α,25-(OH)₂D₃ (20). Neither the conversion of vitamin D₃ to 25-OH-D₃ (19) nor of 25-OH-D₃ to 1α,25-
(OH)\textsubscript{2}D\textsubscript{3} (21) was effected by glucocorticoid treatment; further metabolism of 1α,25-(OH)\textsubscript{2}D\textsubscript{3} may be accelerated, however (22). The calcium transport response of rats to either vitamin D\textsubscript{3} or 25-OH-D\textsubscript{3} is depressed by prior treatment with anticonvulsant (23). Recent data suggests that this effect is the result of inactivation of vitamin D metabolites by an anticonvulsant-induced liver enzyme system (24, 25). The presence of a rachitic factor in plants has been demonstrated (26, 27), but the identity of the substance is not known. Finally, 24-nor-25-OH-D\textsubscript{3} (28) and 19S-19-hydroxy 10(19)-dihydrovitamin D\textsubscript{3} (29) have been reported to possess anti-vitamin D activity. Like 25-N-D\textsubscript{3}, 24-nor-25-OH-D\textsubscript{3} did not impair the bone or intestinal responses to 25-OH-D\textsubscript{3} (28), suggesting that the liver is the site of action for both substances. However, 24-nor-25-OH-D\textsubscript{3} has also been reported to be an active analog of vitamin D (30)\textsuperscript{2} and thus the inhibitory nature of the compound is unclear.

In summary, we have found that 25-N-D\textsubscript{3} antagonizes both the bone and intestinal responses of vitamin D-deficient animals to vitamin D\textsubscript{3}. Since 25-N-D\textsubscript{3} does not inhibit the action of 25-OH-D\textsubscript{3}, and since it causes a build-up of liver vitamin D\textsubscript{3} and a reduction of serum 25-OH-D\textsubscript{3}, the antagonism of the biological responses in rats to vitamin D results from an inhibition of 25-hydroxylation of vitamin D. Furthermore, 25-N-D\textsubscript{3} depresses the intestinal calcium transport response in animals to 1α,25(OH)\textsubscript{2}D\textsubscript{3}, presumably the result of a direct inhibition of the activation (or assembly) of the calcium-translocating machinery. Due to these inhibitory effects, 25-N-D\textsubscript{3} may prove useful clinically in reducing the hypercalcemia of a variety of human disorders such as primary hyperparathyroidism, hypercalcemia of malignancy (pseudohyperparathyroidism), vitamin D toxicity, idiopathic hypercalcemia of infancy, sarcoidosis, and tertiary hyperparathyroidism.

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\textsuperscript{2} Abstract of paper was presented at 59th Annual Meeting of FASEB (Johnson, R. L., and Okamura, W. H. (1975) Fed. Proc. 34, 894). These authors state that 24-nor-25-OH-D\textsubscript{3} is about 50% as active as 25-OH-D\textsubscript{3} in stimulating bone calcium mobilization, and "shows significant activity in mediating intestinal calcium transport."
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