MOLECULAR PHARMACOLOGY, 20:460–462

Bone Resorptive Activity of Side-Chain Fluoro Derivatives of 25-Hydroxy- and 1α,25-Dihydroxyvitamin D_3 in Culture

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Received May 4, 1981; Accepted July 21, 1981

SUMMARY

Three side-chain fluorinated analogues of vitamin D_3 were tested for their bone-resorbing activity on fetal rat forelimb bones *in vitro*. Two fluoro derivatives of 25-hydroxyvitamin D_3 (25-OH-D_3), 24,24-difluoro-25-hydroxyvitamin D_3 and 25-hydroxy-26,26,26,27,27,27-hexafluorovitamin D_3, were compared with 25-OH-D_3. The difluoro compound was approximately 7 times more potent than 25-OH-D_3, and the hexafluoro analogue was approximately 40 times more potent than 25-OH-D_3 in this system. In contrast, the 24,24-difluoro analogue of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) was slightly less potent than 1α,25-$(OH)_2D_3$. The results indicate that the presence of fluoride groups on carbon atoms immediately adjacent to the 25-hydroxyl group enhances the *in vitro* bone-resorbing effects of 25-hydroxyvitamin D_3. On the other hand, fluorination of the 1α-hydroxylated compound on position 24 actually diminished rather than increased bone-resorptive activity.

Fluoride substitution has significant effects on the biological activities of many compounds (1). Mechanisms by which fluoride substitution are postulated to alter biological activity include electronic inductive effects, steric effects, and obstructive halogenation (1). Examples of alteration in biological activity by fluorination can be found in many classes of compounds, most notably the hormonal steroids (2, 3). Recently, a number of fluoro derivatives of compounds in the vitamin D class, including 25-F-D_3,[5] 24-hydroxy-25-fluorovitamin D_3 (24-OH-25-F-D_3), 1α-hydroxy-25-fluorovitamin D_3 (1α-OH-25-F-D_3) and 1α,24-dihydroxy-25-fluorovitamin D_3 (1α,24-$(OH)_2$-25-F-D_3), have been synthesized and tested for biological activity (4–11). It appears from the studies with these 25-fluoro derivatives that the fluorine substituent behaves as a hydrogen atom, supporting previous evidence that the carbon-fluorine bond is stable (12). Furthermore, fluoride substitution on carbon 25 did not prevent further metabolism if the appropriate sites were available (5–8, 10, 11).

Another series of fluorine-substituted compounds have now been synthesized which include the 24,24-difluoro derivatives of 25-OH-D_3 and 1α,25-$(OH)_2D_3$ (13, 14), and 26,27-hexafluoro-substituted 25-OH-D_3 (15). These are of interest because the multiple fluorines could potentially give rise to stronger inductive effects and also greater obstruction. The current study documents the *in vitro* effects of the these three compounds on bone, one of the target tissues of the action of vitamin D.

Fetal rat radii and ulnae were cultured by methods described previously (16, 17). Briefly stated, limbs were dissected from 19-day pregnant Holtzman rats (Holtzman Company, Madison, Wisc.) which had been prelabeled with ^{45}Ca. Bones were initially precultured for 3–6 hr in modified BGJ_b medium (17) without $NaHCO_3$ or albumin. Subsequently, bones were cultured for 48 hr in $NaHCO_3$-buffered modified BGJ_b medium (pH 7.4) containing bovine serum albumin (Reheis Lot 52002), 1 mg/

This work was supported in part by Research Grant AM 11262 from the National Institutes of Health, Program Project Grant AM-14881 from the National Institutes of Health, National Aeronautics and Space Administration Grant NAS-9-15580, United States-Japan Cooperative Grant FJ-6037/5R080 from the National Science Foundation, and by the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation.

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[5] The abbreviations used are: 25-F-D_3, 25-fluorovitamin D_3; 24-OH-25-F-D_3, 24-hydroxy-25-fluorovitamin D_3; 1α-OH-25-F-D_3, 1α-hydroxy-25-fluorovitamin D_3; 1α,24-$(OH)_2$-25-F-D_3; 1α,24-dihydroxy-25-fluorovitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24,24-F_2-25-OH-D_3, 24,24-difluoro-25-hydroxyvitamin D_3; 25-OH-26,26,26,27,27,27-F_6D_3, 25-hydroxy-26,26,26,27,27,27-hexafluorovitamin D_3; 24,24-F_2-1α,25-$(OH)_2D_3$, 24,24-difluoro-1α,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3.

0026-895X/81/030460-03$02.00/0

ml. At the end of the incubation, bones were extracted in 0.1 N HCl, and the ^{45}Ca concentration was determined in aliquots of medium and bone extracts by liquid scintillation spectrophotometry. Results are expressed as follows: percentage of ^{45}Ca released = [medium ^{45}Ca/(medium ^{45}Ca + extract ^{45}Ca)] × 100.

25-OH-D_3 was a generous gift of The Upjohn Company (Kalamazoo, Mich.), and 1α,25-$(OH_2)D_3$ was a generous gift of Hoffmann La Roche (Nutley, N. J.). 24,24-F_2-25-OH-D_3, 24,24-F_2-1α,25-$(OH)_2D_3$, and 25-OH-26,26,26,27,27,27-F_6D_3 were synthesized by previously published techniques (13–15).

Both 24,24-F_2-25-OH-D_3 and 25-OH-26,26,26,27,27,27-F_6D_3 were more potent than 25-OH-D_3 (Fig. 1). According to the ED_{50} for the hexafluoro compound, which was the only analogue to yield maximal responses over the 10^{-9} M–10^{-6} M concentration range, the difluoro compound was approximately 7 times more potent than 25-OH-D_3, and the hexafluoro compound was approximately 40 times more potent than 25-OH-D_3.

The 24,24-difluoro derivative of 1α,25-$(OH)_2D_3$, 24,24-F_2-1α,25-$(OH)_2D_3$, was somewhat less potent than the nonfluorinated analogue (Fig. 2). On the basis of either the ED_{50} for 1α,25-$(OH)_2D_3$ or the potency ratio determined by a standard bioassay method (18), 1α,25-$(OH)_2D_3$ was 1.6 times more potent than the 24,24-difluoro derivative. The potency difference between the two compounds was statistically significant.

The present results show that fluoro substitution on the carbon atoms adjacent to the 25-hydroxy group of 25-OH-D_3 markedly enhances bone-resorptive activity whereas the same adjacent substitution on 1α,25-$(OH)_2D_3$ does not increase and may even slightly decrease activity. This apparent anomalous behavior can probably be explained if one considers that both 25- and 1α-hydroxy functions are important to bone-resorptive activity *in vitro*. It would be expected that, in the derivatives lacking the 1α-hydroxyl group, the importance of the 25-hydroxyl group to binding and activity would be emphasized, whereas in compounds having both groups the importance of the 25-hydroxyl is reduced. Thus, if the adjacent fluoro groups have an inductive effect, it should be emphasized in 25-OH-D_3 and not emphasized in 1α,25-$(OH)_2D_3$. The data presented here are in agreement with this concept; namely, 25-OH-26,26,26,27,27,27-F_6D_3 > 24,24-F_2-25-OH-D_3 > 25-OH-D_3. Furthermore, 24,24-F_2-1α,25-$(OH)_2D_3$ would be expected to be equal in potency to 1α,25-$(OH)_2D_3$ or perhaps slightly more active. Since 24,24-F_2-1α,25-$(OH)_2D_3$ is slightly less active, it might be assumed that the bone receptor-binding affinity is optimized in 1α,25-$(OH)_2D_3$ and that the molecular effects of the fluoro substitution at that site in the molecule could actually be decreasing the over-all binding affinity of the molecule.

The data presented here at first seem inconsistent with *in vivo* data. 24,24-F_2-25-OH-D_3 was found to be equal to 25-OH-D_3 in its *in vivo* bone calcium-mobilizing activity (19), whereas here it was found to be 7 times more potent in bone resorptive activity *in vitro*. This disparity undoubtedly relates at least in part to the further metabolism which the compounds undergo *in vivo*. Since 24,24-F_2-25-OH-D_3 is converted to 24,24-F_2-1α,25-$(OH)_2D_3$ by chick kidney homogenates (14) and is inactive in anephric animals,[6] it is very likely metabolized to 24,24-F_2-1α,25-

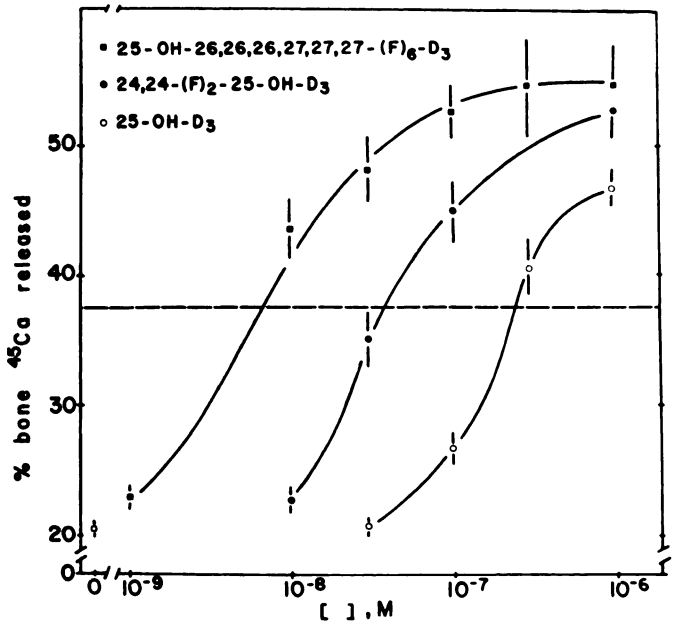

FIG. 1. *Relative bone-resorbing potencies of 25-OH-D_3, 24,24-F_2-25-OH-D_3, and 25-OH-26,26,26,27,27,27-F_6D_3*

Studies were carried out in 48-hr cultures of fetal rat limb bones. Bone resorption was monitored as release of previously incorporated ^{45}Ca (16, 17). Relative potencies, based on the ED_{50} for 25-OH-26,26,26,27,27,27-F_6D_3 (– – –) were as follows: 25-OH-D_3 = 1; 24,24-F_2-25-OH-D_3 = 7; 25-OH-26,26,26,27,27,27-F_6D_3 = 40. Values on the graph are means ± standard errors.

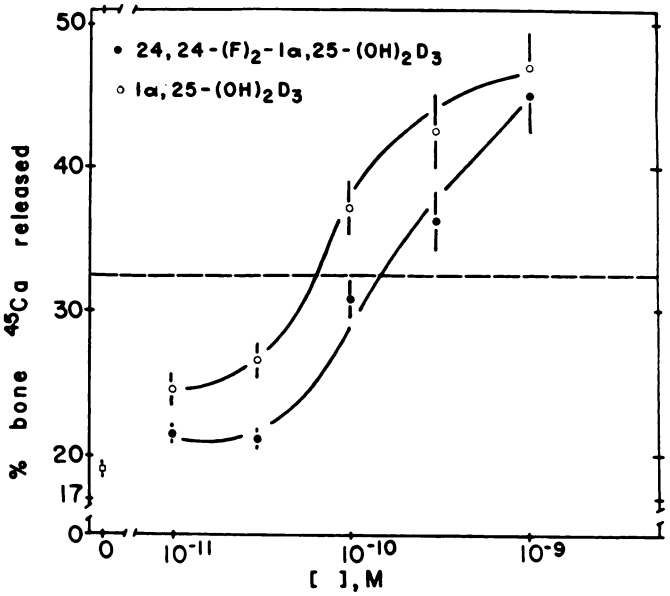

FIG. 2. *Relative bone-resorbing potencies of 1α,25-$(OH)_2D_3$ and 24,24-F_2-1α,25-$(OH)_2D_3$*

Studies were carried out in 48-hr cultures of fetal rat limb bones. Bone resorption was monitored as release of previously incorporated ^{45}Ca (16, 17). Relative potencies, based on the ED_{50} for 1α,25-$(OH)_2D_3$ (– – –), were as follows: 1α,25-$(OH)_2D_3$ = 1; 24,24-F_2-1α,25-$(OH)_2D_3$ = 0.6. Values on the graph are means ± standard errors.

[6] Y. Tanaka and H. F. DeLuca, unpublished data.

$(OH)_2D_3$ *in vivo*. Thus, the observed *in vivo* effects of 24,24-F_2-25-OH-D_3 and 25-OH-D_3 (19) are probably due to their 1α-hydroxylated metabolites, and it is not surprising that the results *in vivo* and *in vitro* are not in agreement.

Recent results indicate that 24,24-F_2-1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$ are approximately equipotent in their ability to compete for binding to the intestinal cytosol receptor (20). This finding is similar to the results reported here for the relative potencies of the two compounds on bone resorption *in vitro*. It is interesting that, in another *in vitro* system, the induction of vitamin D-dependent calcium-binding protein by chick intestine, 24,24-F_2-1α,25-$(OH)_2D_3$ was found to be 4 times more potent than 1α,25-$(OH)_2D_3$ (21). The greater relative potency of 24,24-F_2-1α,25-$(OH)_2D_3$ on calcium-binding protein induction in cultured intestine as compared with ^{45}Ca release from fetal rat bone or intestinal receptor binding could conceivably arise if 1α,25-$(OH)_2D_3$ undergoes inactivation by the cultured intestine, possibly by 24-hydroxylation or steps affecting the side-chain which do not occur in the bone cultures or the receptor system.

In conclusion, the studies indicate that fluoro-substitution can markedly enhance the *in vitro* bone-resorbing activity of compounds in the vitamin D series but that the effect will not be apparent if the activity of the compound is already optimized by substituents elsewhere in the molecule. Since 24,24-F_2-25-OH-D_3 is not converted to 24,25-$(OH)_2D_3$ (22), the results provide evidence that 24-hydroxylation is not required for the *in vitro* bone-resorbing effects of the vitamin D compounds.

ACKNOWLEDGMENT

We thank Thalia Mavreas for her excellent technical assistance.

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