Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1041–1046

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THREE-DIMENSIONAL STRUCTURE-FUNCTION RELATIONSHIP OF VITAMIN D: SIDE CHAIN LOCATION AND VARIOUS ACTIVITIES

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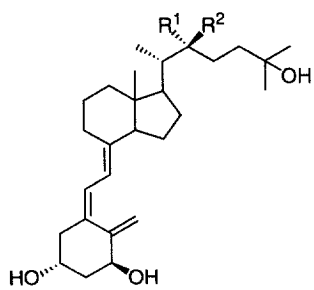
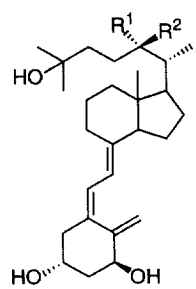
Received 18 January 1999; accepted 26 February 1999

Abstract: The various biological activities of side-chain mobility restricted analogs, four diastereomers at C(20) and C(22) of 22-methyl-1α,25-dihydroxyvitamin D_3, were evaluated. The relationship between structure and the various activities of the analogs was discussed in terms of the active space region concept that we previously suggested. © 1999 Elsevier Science Ltd. All rights reserved.

The calcium-regulating hormone, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, **1**), is also involved in such basic functions of the cell as regulation of proliferation, differentiation and the immune response.[1] 1,25-$(OH)_2D_3$ **1** is believed to carry out all these functions by binding to the vitamin D receptor (VDR), a member of the nuclear receptor superfamily, and regulating the expression of target genes. It is now well documented that the transactivation function of members of the nuclear receptor super family is highly dependent on the conformation of a small part of its ligand binding domain called AF2 and that three-dimensional structure of the ligand is crucial for determining its conformation.[2] Thus, for a better understanding of the mechanism of gene expression, it is important to determine the three-dimensional structure of the ligand responsible for binding to the receptor and for transactivation function.



1: R^1=H, R^2=H
3: R^1=Me, R^2=H
4: R^1=H, R^2=Me



2: R^1=H, R^2=H
5: R^1=H, R^2=Me
6: R^1=Me, R^2=H

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PII: S0960-894X(99)00129-8

Figure 1. Stereoview of the side-chain regions of $1,25(OH)_2D_3$ (**1**) and 20-epi-$1,25(OH)_2D_3$ (**2**). The most stable conformations of the two vitamins (**1** and **2**), which are represented as ball and stick, are superimposed. Dots show the regions where the 25-oxygen of the side cahin of **1** and **2** can reach: yellow, $1,25(OH)_2D_3$ (**1**); cyan, 20-epi-$1,25(OH)_2D_3$ (**2**). A and G are regions for **1** and EA and EG are regions for **2**.

In a series of studies on conformationally restricted vitamin D analogs, we proposed the side chain conformation of vitamin D responsible for binding to VDR. [3] In the above studies, we categorized the regions occupied by a 25-hydroxy group of vitamin D into four groups, A, G, EA, and EG on the basis of a conformational analysis of $1,25\text{-}(OH)_2D_3$ **1** and 20-epi-$1,25\text{-}(OH)_2D_3$ **2** (Figure 1).[4] We subsequently designed and synthesized analogs (**3**-**6**) with side chain conformations restricted to one of these four regions and evaluated their VDR binding affinity.[3] The order of VDR affinity was found to be EA>A>G>EG in terms of the spatial region. From these results, we proposed that $1,25\text{-}(OH)_2D_3$ (**1**) binds to VDR when its side chain is directed to the A region and that the 20-epimer **2** binds to VDR when its side chain is directed to the EA region. We also demonstrated[5] that our concept of an active side chain region is applicable to the majority of potent vitamin D analogs reported.[6] In the present study, we evaluated the potency of four conformationally restricted analogs (**3**-**6**) in various functions of vitamin D. The results provide further insight into the relationship between the three-dimensional structure of vitamin D and its function.

Evaluation of biological activities

The biological activities of side-chain restricted analogs were evaluated in comparison with a natural hormone **1**. The discussion of the results focuses on the spatial region and activity relationship.

Transcriptional activity. Transcriptional stimulation was analyzed using transient transfection assays with fibroblastic mouse NIH3T3 cells which were transfected with a luciferase reporter which encodes three copies of the mouse osteopontin vitamin D responsive element (VDRE).[7] The results are shown in Table 1. Two pairs of 22-epimers (**3**/**4** and **5**/**6**) exhibited distinct activities. In the pair of analogs (**3** and **4**) with a natural C(20) configuration, 22*S*-isomer **4** was found to be 22 times more potent than 20*R*-isomer **3**. The difference in potency was much more striking between the pair of 20-epi analogs **5** and **6**, 22*R*-isomer **5** being approximately 3000 times more potent than the 22*S*-isomer **6**. Transcriptional activity, assayed using a chimera vector composed of a GAL4 DNA binding domain and a VDR ligand-binding domain (GAL4-VDR)[8] which was transfected in CV-1 cells showed a similar result (data not shown). These results indicate that the A and EA regions are responsible for transcriptional activation of VDR. It should be noted that transcriptional activity and VDR affinity are similar in the 20-normal analogs (22% and 33%, respectively, for **4** and 1% and 1.7~2%, respectively for **3**) but in the 20-epi analogs the transcriptional activity is amplified 3~9-fold compared with VDR affinity (10000 vs. 1100~2000 for **5** and 3% vs. 0.4~1% for **6**). The augmented transcriptional activity found in the 20-epi analogs may be partly ascribed to the extremely low affinity of these compounds for vitamin D binding protein (DBP) in serum, as the assay system includes fetal bovine serum (FBS) and the concentration of free vitamin D is believed to be higher with lower DBP-binding substrates than with higher DBP-binding substrates. However, this is not true with 20*R*,22*R*-analog **3** which shows still lower transcriptional activity even though its DBP affinity is considerably low. The conformation of VDR liganded with 20-epivitamin D was reportedly different from that liganded with a natural ligand in a proteolysis study of liganded VDR.[9] Thus, the conformation of AF-2 in VDR liganded with 20-epivitamin may be different from that in VDR liganded with a natural hormone. The different conformation of AF-2, and hence the different interaction of AF-2 with the co-activator could be a cause of the augmented transcriptional activity of 20-epi analogs. Transcriptional activity was found to decrease in the following order, EA>A>EG>G in terms of the side-chain region, and in the last two, the order between VDR affinity and transcriptional activity was reversed.

In vitro activity. Differentiation of HL-60 cells. Potency in inducing differentiation of cells was evaluated using human promyelocytic leukemia (HL-60) cells.[10] As shown in Table 1, the potency difference between the 22-epimer pairs was again obvious. Only one of the 22-epimeric pairs showed a high potency. This was the 22*S*-isomer **4** in 20*R*-vitamins and the 22*R*-isomer **5** in 20*S*-vitamins, which occupy the A and EA regions, respectively, and are highly active in the induction of differentiation. The activity ratio of **4** and **3** was 80:1 and that of **5** and **6** was 1500:1. Notably, **4** retained differentiating activity comparable to that of **1**, even though its transcriptional activity was only 22% of **1**. In evaluating cell differentiating activity, the cells were cultured with a substrate for 4 days. Thus, the lifetime of the substrate in the cells may have some effect on potency. The 22-methylated ligand **4** may have a longer half-life than that with a natural ligand, as the methyl at C(22) may cause steric hindrance in 23-hydroxylation, which may cause the amplified effect of the 22-methyl analog **4**. The 20-epi analogs **5** and **6** again showed augmented potency and this potency was slightly higher than that found in transcriptional activity.

Antiproliferation of MCF-7 cells and keratinocytes. The potency of the two 20-epi-22-methyl analogs **5** and **6** in inhibiting proliferation of human breast cancer cells (MCF-7) and primary cultures of human foreskin keratinocytes was evaluated.[11] Here as well, the difference in potency between 22*R*- and 22*S*-isomers was distinct. The results indicate that the high potency of vitamin D super agonists with 20-epi configuration appears only when their side chain is capable of occupying the EA region.

Table 1. Side-chain region and biological activities of **1-6**[a]

		binding affinity[b]			gene trans.[c]	in vitro activity[c, d]			in vivo activity[e]	
compd.	side-chain region	VDR (porcine)	VDR (bovine)	DBP (rat)	OPN[f]	HL-60	MCF-7	Kerat.	BCM	ICA
1	A & G	100	100	100	100	100	100	100	100	100
3	G	1.7	2	0.45	1	1.3	-	-	32	25
4	A	33	33	67	22	100	-	-	26	50
5	EA	2000	1100	<0.25	10000	12000	15000	9000	220	51
6	EG	1	0.4	<0.25	3	8	70	60	5	<1
2	EA & EG	-	500	-	4000	-	-	-	-	-

[a] Activities are presented as % effect of $1,25\text{-}(OH)_2D_3$ **1**. [b] See reference 3d. [c] The relative activities were calculated at ED_{50}. [d] HL-60 cell differentiation was determined by an NBT reduction assay. Proliferation was assessed by measuring [^{3}H]-thymidine incorporation. [e] BCM was determined by the rise in serum calcium and ICA was measured using the everted intestinal sac method as described earlier[12b]. The relative activities were calculated by the increased calcium concentration compared to the vehicle. [f]osteopontin.

In vivo activity. Bone calcium mobilization (BCM) and intestinal calcium transport (ICA). In vivo calcemic activity was evaluated by BCM and ICA in vitamin D deficient rats (Table 1).[12] Notably, **5** shows high potency in in vivo BCM, though it has a low affinity for the transport protein (DBP) of vitamin D. The same trend has also been reported in the activities of other 20-epi vitamin D analogs. However, the same was not true in ICA. It is also noteworthy that the analog **5** was more potent than natural hormone **1** in all activities tested aside from two, DBP affinity and ICA. Thus, it is assumed that DBP affinity is necessary for in vivo ICA activity. DBP is believed to be important for ensuring that vitamin D has a long survival-time in the blood. Thus, a long period of survival in the blood is likely necessary for ICA activity to occur but not for BCM. It is also interesting that 22-epimers **3** and **4** of 20*R*-analogs show similar in vivo calcemic activity, whereas the two isomers showed distinct potency in gene transcription and cell differentiating activities.

Conclusion

We explicitly showed the relationship between the three-dimensional structure of vitamin D and the various functions associated with it using logically designed four conformationally confined analogs, **3**-**6** as powerful probes. The regions EA and A are shown to be important in transcriptional and in vitro cell-differentiating activities. However, these activities were not simply related to their VDR affinity. AF-2 conformation induced by ligands, lifetime in the cells, and affinity for DBP were proposed to affect the in vitro potencies. In vivo actions were much more complicated to explain, involving multiple other factors besides DBP affinity. Thus, although EA was still the most active region, the A- and G-region analogs **4** and **3**, which showed big differences in in vitro activities, nevertheless showed similar ICA and BCM activities. From these studies, we obtained a rough idea of the three-dimensional structure required for VDR binding and gene transcription. Further studies, such as a synthesis of rigid VDR ligands that imitate the suggested three-dimensional structure, and a docking of the ligands **3**-**6** in VDR constructed using homology modeling from crystal structures of other nuclear receptor members,[13] are needed to confirm our three-dimensional structure-function theory. Those studies are currently underway in our laboratory.

Acknowledgment

We are grateful to Dr. L. Binderup of Leo Company for providing the compound **2**.

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