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The C_{19} Position of 25-Hydroxyvitamin D_3 Faces Outward in the Vitamin D Sterol-Binding Pocket of Vitamin D-Binding Protein

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Abstract—The radiolabeled affinity and photoaffinity analogues of 25-hydroxyvitamin D_3 (25-OH-D_3) with probes at the C-19 position failed to specifically label the 25-OH-D_3-binding pocket of vitamin D-binding protein (DBP). However, a hybrid analogue, with a bromoacetate affinity probe and a photoaffinity probe at C_3-OH and C_{19} positions, respectively, specifically labeled the ligand-binding pocket, suggesting that C_3-OH points towards the 'inside' of the binding cavity while the C_{19} position faces away from it.

Functions of vitamin D binding protein (DBP) include binding and organ-specific transportation of vitamin D and its metabolites, for example 25-hydroxyvitamin D_3 (25-OH-D_3) and 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], for their biological activation; and selective scavenging of actin-monomers from blood to prevent clogging of arteries during cell-injury and death.[1,2] In addition, DBP binds fatty acids and chemotactic agents. Recently a post-translationally modified form of DBP was shown to be a potent activator of macrophages and osteoclasts, and a strong inhibitor of tumor in mice.[3,4]

Biological function of a protein is critically dependent on the three-dimensional structures of its ligand and its ligand-binding pocket. Therefore, identification of contact points of a ligand inside the binding pocket is important towards establishing structure–function relationship between the ligand and its binding protein.

Ligand-mimics, with affinity/photoaffinity probes at different parts of the parent ligand, are important in identifying contact points in different areas within the binding pocket. In the past, several affinity and photoaffinity analogues of 25-OH-D_3 and $1,25(OH)_2D_3$, containing the affinity/photoaffinity probes at C_1, C_3, C_6, and C_{11} positions of the parent sterols have been developed to probe the ligand-binding pockets of DBP[5–12] and vitamin D receptor, VDR, the nuclear receptor for $1,25(OH)_2D_3$.[13–21] Moreover, recently we developed a synthetic method for the functionalization of the $C_{10–19}$-*exo*-cyclic methylene of 25-OH-D_3, and synthesized affinity and photoaffinity analogues of 25-OH-D_3 [(**A**) and (**B**), respectively] (Fig. 1), in which the cross-linking probes are attached to the C_{19}-position via a short tether.[22]

However, covalent labeling of DBP by (**A**) and (**B**) required the availability of the radiolabeled counterparts of these compounds. In the present study we synthesized the ^{3}H-labeled counterparts of (**A**) and (**B**) (Fig. 1) and attempted to label DBP.

Incubation of two samples of DBP (10 μg) with **^{3}H-(A)** (10,000 cpm, sp. ac 250 mCi/mM) at 4 °C, in the absence or in the presence of 25-OH-D_3 (one μg), followed by electrophoresis and autoradiography produced faintly labeled DBP bands of equal intensity in both cases, demonstrating the nonspecific nature of labeling (results not shown). On the other hand, incubation of DBP samples (10 μg each) with **^{3}H-(B)** (10,000 cpm, sp. ac 250 mCi/mM), in the absence or in the presence of 25-OH-D_3 (1 μg) at 4 °C in the dark, followed by exposure to light (254 nm, Hanovia mercury arc lamp, 2 cm height, 1 min) did not label the protein with or without an additional 25-OH-D_3 (results not shown).

Affinity labeling requires a nucleophilic amino acid within the bonding distance of the carbon atom with the

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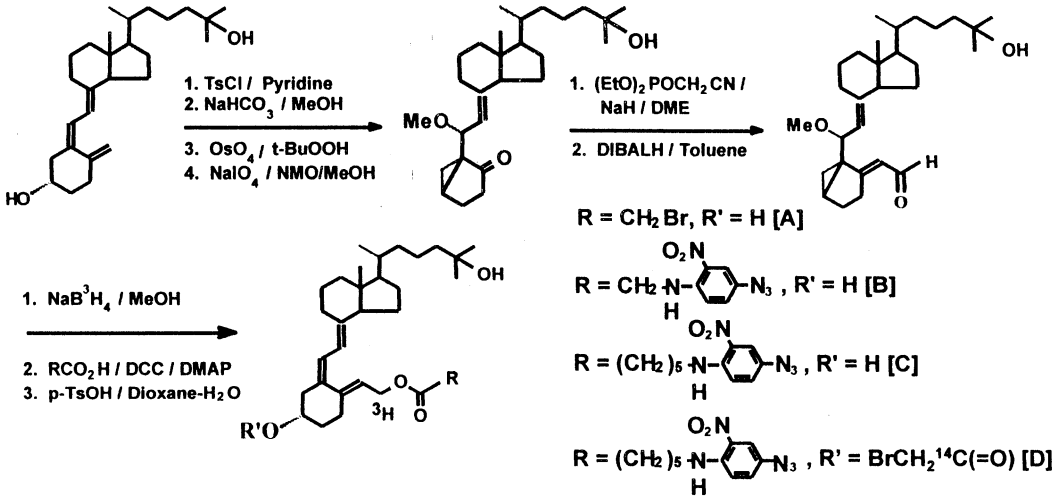

Figure 1. Scheme for the synthesis of radiolabeled compounds (**A**)–(**D**).

leaving group. Hence, inability of **^{3}H-(A)** to specifically label DBP suggested the absence of such an amino acid in the vicinity of –OCO–CH_2–Br in **^{3}H-(A)**.

In contrast, photoaffinity labeling does not require such a juxtaposition. Therefore, failure of **^{3}H-(B)** to photoaffinity label DBP suggested the absence of any part of the protein in close proximity of the azidonitrophenyl group in **^{3}H-(B)**.

We hypothesized that elongating the linker between C_{19} of the steroid and the azidonitrophenyl group of **^{3}H-(B)** might allow the photo-probe to come close to an amino acid in DBP. Hence, we synthesized **^{3}H-(C)** with a longer tether than **^{3}H-(B)** (Fig. 1). But **^{3}H-(C)** failed to label DBP in the absence or in the presence of an excess of 25-OH-D_3 similar to **^{3}H-(B)**.

In the case of photoaffinity labeling experiments with **^{3}H-(B)** and **^{3}H-(C)**, all the radioactivity appeared at the bottom of the polyacrylamide gel (dye front). This observation raised the possibility that the nitrene-intermediate, generated by the photolysis of **^{3}H-(B)** and **^{3}H-(C)**, might form covalent bond with 25-OH-D_3 when an excess of 25-OH-D_3 was added to the reaction mixture. However, a very similar result was obtained with reaction mixtures where additional 25-OH-D_3 was absent. Furthermore, compounds **(B)** and **(C)** bound specifically and strongly to DBP, as shown by a competitive binding assay (Table 1), suggesting that compounds **(B)** and **(C)** share the same binding pocket of DBP with 25-OH-D_3. Therefore, inability of **^{3}H-(B)** and **^{3}H-(C)** to covalently label DBP strongly suggested that the azidonitrophenyl group in **^{3}H-(B)** and **^{3}H-(C)** might not be close to any part of the protein while these molecules are 'seated' inside the vitamin D sterol-binding pocket of DBP.

It was shown earlier that 25-hydroxyvitamin D_3-3β-2′-bromoacetate covalently modifies the vitamin D sterol-binding sites of DBP[9,12] and VDR.[23] Based on this information, we recently synthesized a hybrid analogue of 25-OH-D_3 containing an affinity and a photoaffinity analogue [(**D**), Fig. 1], and isolated a vitamin D receptor associated factor (23).

Incubation of **^{14}C-(D)** (5,000 cpm, sp. ac. 8.4 mCi/mM) with a sample of DBP (10 μg) covalently labeled the protein (Fig. 2, lane 1), and labeling was significantly reduced when the incubation was carried out in the presence of an excess of 25-OH-D_3 (one μg) (Fig. 2, lane 2). These results showed that **^{14}C-(D)** labeled the vitamin D sterol-binding pocket of DBP.

We showed earlier that (**A**) and (**B**) specifically bind to DBP in a competitive binding assay.[22] A similar assay was carried out for compounds (**C**) and (**D**). Briefly samples of human serum DBP (0.01 μg) were incubated with ethanolic solutions (10 μL) of ^{3}H-25-OH-D_3 (3000 cpm, 20 Ci/mM) and various concentrations of either 25-OH-D_3 or the analogues at 4 °C in the dark for 12 h. followed by incubation with Dextran-coated charcoal (4 °C, 20 min), centrifugation and counting of radioactivity of the supernatants. Nonspecific binding was determined by incubating DBP samples with 25-OH-D_3 (1 μg).

Concentrations of 25-OH-D_3 or the analogues at 50% specific-binding (Table 1) showed that all the analogues possessed specific-binding for DBP. It is noteworthy that compound (**D**), despite having the least binding-affinity for DBP, covalently modified the 25-OH-D_3-binding pocket of DBP. This was a reflection of the kinetic process involved in the affinity labeling of DBP by (**D**).

We have shown earlier that the vitamin D sterol-binding pockets of DBP[26] and vitamin D receptor (VDR), a

Table 1. Concentrations of 25-OH-D_3 or analogues at 50% specific-binding to DBP (shown in parentheses)

25-OH-D_3	0.008 nm
(**A**)	0.011 nm
(**B**)	0.014 nm
(**C**)	0.11 nm
(**D**)	0.42 nm

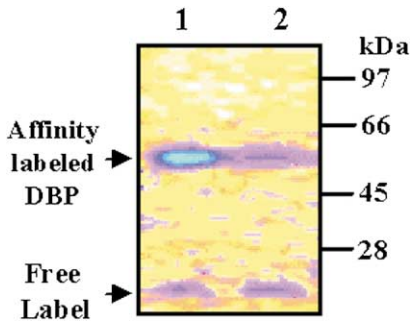

Figure 2. Affinity labeling of human DBP with **^{14}C-(D)**. Lane 1. DBP + **^{14}C-(D)**; lane 2: DBP + **^{14}C-(D)** + 25-OH-D_3 (excess); positions of MW marker proteins are denoted on the right.

nuclear vitamin D receptor,[20,21] are sterically restricted, in sharp contrast with several cases where a ligand 'tumbles' or 'spins' inside the binding pocket of its cognate binding protein/receptor. For instance, it was shown that estradiol-3-bromoacetate and estradiol-17-bromoacetate specifically labeled the same His residue in E_2-HSD.[24] Similarly it was reported that 17-alkyl-α-carbonyl-halo esters or amides of estradiol specifically labeled four[4] Cys residues in the estrogen receptor-binding pocket.[25]

In the case of DBP, **^{3}H-(A)**, **^{3}H-(B)**, and **^{3}H-(C)** failed to specifically label the vitamin D sterol-binding pocket of DBP, while **^{14}C-(D)** successfully labeled it. This observation emphasized that these compounds, once inside the binding pocket, cannot rotate or tumble, otherwise we would expect covalent labeling of DBP with **^{3}H-(A)**, **^{3}H-(B)**, and **^{3}H-(C)** also.

Furthermore, successful labeling with **^{14}C-(D)** suggested that the bromoacetate group in this compound, and in the same token C_3-OH of 25-OH-D_3 points towards the 'inside' of the binding pocket. This is very similar to the hormone-binding pocket VDR, where the hormone molecule [1,25$(OH)_2D_3$] enters the binding cavity with the 3-OH group pointing inside the cavity.[20,21,27] However, in the case of VDR, the hormone molecule is completely enveloped by different parts of the protein inside the binding pocket.[27] In contrast, results described here strongly suggest that the C_{10-19}-methylene of 25-OH-D_3 molecule projects either towards the 'opening' of the binding cavity or it actually stays outside the cavity.

In conclusion, we have employed several affinity and photoaffinity analogues of 25-OH-D_3 to probe the vitamin D sterol-binding pocket of DBP. Our studies showed that this pocket is sterically restrictive, so that tumbling of the ligand inside this pocket is largely prohibited. We have also showed that inside this pocket, orientation of 25-OH-D_3, the most important ligand of DBP, is such that it's A-ring and the C_3-OH group points towards the inside the cavity, while the C_{19}-*exo*-cyclic methylene group projects outside the cavity.

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