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Affinity Labeling of the Nuclear Vitamin D Receptor with Nonsteroidal Alkylating Agents

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Abstract—Synthesis of an affinity alkylating non steroidal mimic of 1α,25-dihydroxyvitamin D_3 and its radiolabeled counterpart is presented. We also report the affinity labeling of the VDR-ligand binding domain (VDR-LBD) with this analogue.
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1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2$ D_3) is a multifunctional *seco*-steroid. Its biological properties include calcium and phosphorus homeostasis, growth and maturation control of a broad range of normal and malignant cells, and immune-regulation. Therapeutic value of $1,25(OH)_2$ D_3 has been amply demonstrated in many diseases including cancers of various organs, leukemia, psoriasis, osteoporosis, autoimmune diabetes, etc.[1] However, $1,25(OH)_2$ D_3 causes hypercalcemia in rodents leading to soft-tissue calcification, loss of bodyweight, and death. In humans $1,25(OH)_2$ D_3 induces hypercalcemia, which is manifested in kidney stones, hypercalciuria, and related diseases.[2]

Adverse side effects of $1,25(OH)_2$ D_3 have garnered a strong interest in developing analogues of $1,25(OH)_2$ D_3 with intact beneficial effects and reduced toxicity. During the past several years numerous vitamin D analogues have been synthesized and their biological activity evaluated.[3] Although a number of such analogues, with significant separation between antiproliferative effects and systemic toxicity, have been developed, no information is available to date that demonstrates predictable trends in modification of analogues that will (i) have enhanced antiproliferation and decreased calcemic properties, (ii) show tissue-specific effects, and (iii) display antihormone activities.

Mechanism of $1,25(OH)_2$ D_3-action closely parallels estrogen, another steroid hormone. Thus, strong and specific interaction between $1,25(OH)_2$ D_3 and its nuclear receptor, vitamin D receptor (VDR) promotes a conformational change in the latter and allosteric binding to retinoid X receptor (RXR), and binding of the VDR-$1,25(OH)_2$ D_3-RXR to several co-activators and to the upstream promoter region of the vitamin D-regulated genes, initiating gene expression.[4] The key to such highly specific molecular interactions, leading to observed biological activities, lies in the three-dimensional architectures of the interacting molecules.[5]

In the case of estrogen receptor (ER), a nuclear receptor of the same family as VDR, several artificial nonsteroidal ligands, having drastically different structures than the parent hormone, have been developed. Some of these nonsteroidal mimics have shown tissue-specific activity and antihormonal effects with high therapeutic values.[6] It has been demonstrated that some of these compounds, for example, tamoxifen and raloxifen stabilized the nuclear estrogen receptor by causing drastically different conformational changes in the estrogen receptor (ER) than the parent hormone, particularly in the relative positioning of the C-terminal helix-12 (H-12); and such changes are reflected in their unique biological properties.[7] On the other hand, nonsteroidal anticancer agents flutamide and biculatamide are known to act as androgen receptor (AR) ligands, and

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stabilize AR differently than the parent hormone.[8] Similarly a novel vitamin D analogue has been shown to act as a VDR antagonist.[9] Recently Moras's group carried out X-ray diffraction analysis of VDR, complexed with the hormone and two noncalcemic side-chain analogues, and showed that these compounds stabilized VDR stronger than the hormone without causing drastic conformational changes in VDR.[10]

Boehm et al. recently reported that a class of biphenyl compounds (Fig. 1) mimicked the actions of 1,25(OH)$_2$ D$_3$, such as anti-proliferation/pro-differentiation of several normal and malignant cells and regulation of vitamin D-controlled genes, without eliciting hypercalcemia in mice.[11] Furthermore, one compound in this group (LG190178) showed strong activity in reducing skin xenograft human prostate tumor in nude mice.[12] This provided a unique opportunity to study the interaction between this class of non-vitamin D compounds with VDR.

We initiated the present study to develop potential affinity alkylating derivatives of LG190178 (Fig. 1) to determine the spatial orientation of these compounds inside the vitamin D-binding pocket of VDR by identifying the contact points between these compounds and peptide backbone of VDR. In this communication we report the synthesis of an affinity alkylating derivative (**H**) and its radiolabeled counterpart. We also report affinity labeling of VDR-ligand binding domain (VDR-LBD) with this analogue.

Our original synthetic strategy included the esterification of the ketone (**A**) to obtain the corresponding bromoesters. The addition of one equivalent of bromoacetic acid to the ketone (**A**) produced a separable mixture of two bromoesters. However, sodium borohydride resulted in the reduction of the carbonyl, as well as hydrolysis of the ester to produce the triol (**B**) (Scheme 1).

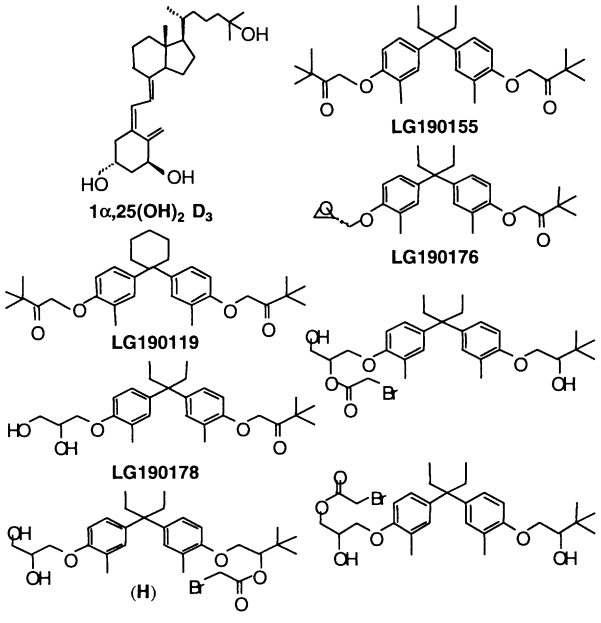


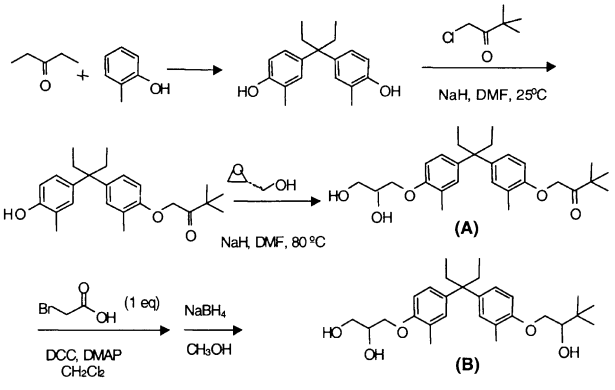
Figure 1. Structures of 1,25(OH)$_2$ D$_3$, its non-steroidal mimics and their affinity analogues.

We decided to carry out the protection of the hydroxyl groups of the ketone (**A**) as TBDMS ethers. The mono- (**C**) and di-protected (**D**) derivatives were obtained by standard procedure. Esterification of the mono-TBDMS ether (**C**) with bromoacetic acid afforded the bromoacetate. But attempted deprotection of the silyl group largely produced the diol (**E**). Alternatively, borohydride reduction, bromoacetic acid esterification and TBAF deprotection of the primary hydroxyl group produced the triol (**B**) (Scheme 2).

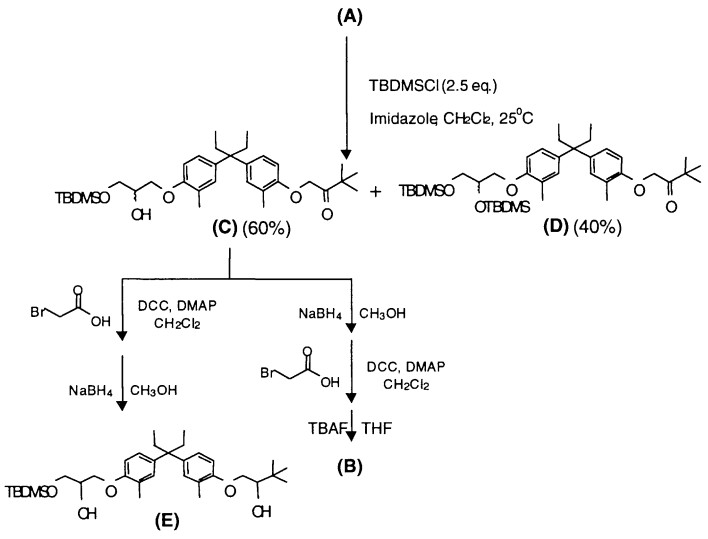
The di-protected ketone (**D**) was quantitatively reduced with $NaBH_4$ in methanol, and the resulting alcohol (**F**) was esterified with bromoacetic acid to give the monoester (**G**). Deprotection of (**G**) with TBAF resulted in the hydrolysis of the ester exclusively producing the triol (**B**). However, deprotection of the hydroxyl groups with 10% HF in dichloromethane efficiently produced the desired bromoacetate (**H**) (Scheme 3).

Radiochemical synthesis of (**H**) was accomplished by replacing bromoacetic acid in Scheme 3 with [1-^{14}C]bromoacetic acid (Sigma Chemical Co., Milwaukee, WI, USA, sp. ac. 10.8 mCi/mmol). A parallel reaction with unlabeled bromoacetic acid, spiked with [1-^{14}C]bromoacetic acid, was carried out to purify (by TLC), characterize and determine radiochemical purity of the labeled products (**G**) and (**H**).

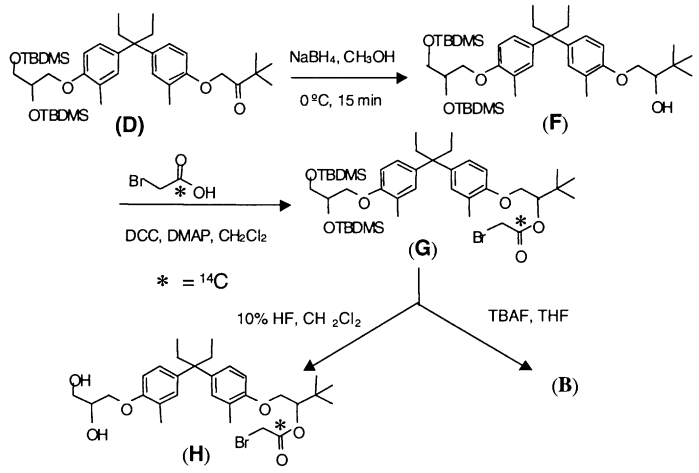
Incubation of two samples of VDR-LBD (103–427, $M_{r\text{app}}$ 39,000)[13] (10 μg, 0.26 nmol, in a buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM DTT, 0.1% Triton X, pH 8.3) at 0 °C for 2 h with **[^{14}C]-H** (Sp. activity 8.4 mCi/mmol, 5000 cpm, 0.6 nmol in EtOH) covalently labeled the protein, as evidenced by the SDS-PAGE analysis and radioactive scanning (Fig. 2, Lane 1). On the other hand, when the incubation was carried out in the presence of an excess of 1,25(OH)$_2$ D$_3$ (1 μg, 2.4 nmol), labeling was significantly reduced (Fig. 2, Lane 2). These results strongly suggested that **[^{14}C]-H** specifically labeled the 1,25(OH)$_2$ D$_3$-binding pocket of VDR-LBD. It should be noted that labeling was not completely obliterated by co-incubation with 1,25(OH)$_2$



Scheme 1.



Scheme 2.



Scheme 3.

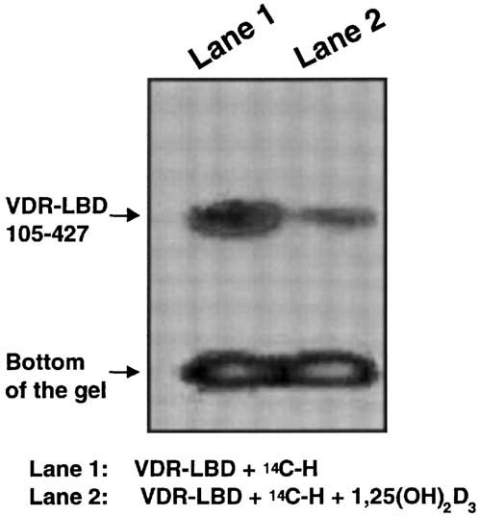


Figure 2. Affinity labeling f VDR-LBD (105-427) with ^{14}C-H.

D_3. This is to be expected because we could not employ a large excess of 1,25(OH)$_2$ D_3 due to insolubility problem. In addition, the labeling process is rapid, so that the unlabeled VDR-LBD is always expected to be contaminated with a finite amount of the labeled protein.

According to the published report all the nonsteroidal 1,25(OH)$_2$ D_3-mimics (Fig. 1)[11] and certain A-ring modified steroidal 1,25(OH)$_2$ D_3-mimics[14] with drastically reduced VDR-binding affinities (than the parent hormone) displayed significant 1,25(OH)$_2$ D_3-like activities in vitro. Results of our studies described in this communication strongly suggested the mediation of VDR in the 1,25(OH)$_2$ D_3-like activities of LG190178, one of the nonsteroidal mimics. Currently we are in the process of identifying the contact point/points between LG190178 and VDR-LBD.

It was mentioned earlier that nonsteroidal 1,25(OH)$_2$ D_3-mimics showed poor VDR-binding activity, which might compromise their bioavailability in vivo. We

hypothesize that (**H**), the affinity labeling derivative of LG190178 and similar reagents might cross-link to endogenous VDR in an in vivo system, and make this analogue more bioavailable for desired biological activities. Therefore these affinity analogues could potentially have better therapeutic index than the parent compounds.

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