Bioorganic & Medicinal Chemistry Letters 20 (2010) 1712–1717

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

LXXLL peptide mimetics as inhibitors of the interaction of vitamin D receptor with coactivators

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ARTICLE INFO

Article history:
Received 3 December 2009
Revised 7 January 2010
Accepted 8 January 2010
Available online 21 January 2010

Keywords:
Vitamin D
Antagonist
Co-factor
Nuclear receptor
Non-peptide
Molecular design
Protein interaction inhibitor

ABSTRACT

Suppression of vitamin D receptor (VDR)-mediated transcription is expected be of therapeutic value in Paget's disease. Once an agonist activates VDR, recruitment of additional coactivator proteins is essential for transcription. Neither non-secosteroidal VDR antagonists nor non-peptide coactivator binding inhibitors for VDR have been reported so far. Based on the X-ray structure of VDR and an LXXLL-containing peptide fragment of the coactivator (where L is leucine and X is any amino acid), which adopts a partially α-helical conformation, benzodiazepine molecules were rationally designed as non-peptide coactivator mimetics. TR-FRET assay showed that the synthesized compounds inhibited the interaction between VDR and a coactivator peptide fragment. Compound **2** showed an IC_{50} of 20 μM. Compound **2** also inhibited VDR-mediated transcription, and this activity was independent of the concentration of co-existing agonist. Furthermore, compound **2** did not inhibit estrogen receptor α-mediated transcription, indicating that it is not a non-selective inhibitor of other nuclear receptors.

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is associated with regulation of calcium homeostasis, bone mineralization, proliferation and differentiation of various types of cells, and immune modulation.[1–3] These biological effects of $1,25(OH)_2D_3$ are known to be elicited by binding of $1,25(OH)_2D_3$ to the ligand binding domain (LBD) of vitamin D receptor (VDR), a member of the nuclear receptor (NR) superfamily. This binding results in heterodimer formation with retinoid X receptor (RXR), which enables high-affinity binding to the vitamin D-responsive element (VDRE) sequence within, and subsequent transcription of vitamin D target genes, including the genes encoding bone proteins osteocalcin and osteopontin, and a metabolic enzyme, 25-hydroxyvitamin D_3-24-hydroxylase.[4,5] Chemical modification of $1,25(OH)_2D_3$ has yielded a number of secosteroidal agonists that bind to the LBD of VDR. Some of them have been reported to elicit a higher ratio of desirable effects to unwanted calcium mobilization effects.[1] One of these analogs, MC903 is currently in use as a topical treatment for mild to moderate psoriasis.[6] In addition, non-secosteroidal VDR agonists[7–10] with greater stability, easier synthesis, and reduced calcium-raising effects have been developed. Some of them do not bind to serum vitamin D binding protein,[7] a property that has been correlated with lower calcemic potential in vivo.[11]

Paget's disease of bone (PD) is characterized by an increased number of osteoclasts and excessive bone resorption in focal areas.[12] The excessive breakdown and formation of bone tissue that occur with PD cause weakened bones, resulting in bone pain, arthritis, deformities and fractures. Osteoclast precursors from patients with PD are hyperresponsive to $1,25(OH)_2D_3$.[13] This hyperresponsiveness may promote osteoclast formation and play a role in the pathogenesis of PD. Therefore, VDR antagonists are expected to be of therapeutic value. In fact, VDR antagonists were reported to suppress excessive bone resorption and osteoclast formation.[13,14] Many vitamin D analogs have been synthesized so far, but almost all of these compounds are VDR agonists. Only a few families of secosteroidal VDR antagonists that bind to LBD have been reported,[15–21] and no non-secosteroidal VDR antagonists are known.

As an alternative approach to block the VDR signal, we focused on the interaction between $1,25(OH)_2D_3$-activated VDR and coactivator proteins. Once the ligand-activated VDR/RXR heterodimer binds to VDRE on a target gene, the complex recruits additional coactivators, including vitamin D receptor interacting proteins

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doi:10.1016/j.bmcl.2010.01.079

(DRIP) and the steroid receptor coactivator (SRC) family of proteins, which are essential for transcriptional activation of the responsive genes.[22,23]

The coactivator proteins of NRs, including VDR, possess multiple copies of a general amino acid sequence motif, that is, a conserved LXXLL motif (where L is leucine and X is any amino acid). Extensive studies have shown that this short LXXLL sequence is necessary and sufficient for the binding of these proteins to NRs and for enhancing transcriptional activity.[24] In the search for inhibitors of this interaction, various short peptide derivatives based on the LXXLL sequence have been shown to disrupt the interactions of coactivators with NRs,[25–27] including VDR.[28,29] However, there have been only a few reports of non-peptide inhibitors designed to bind to this surface region of NR and block the binding of coactivators (Fig. 1).[30–36] In particular, no non-peptide inhibitor of VDR-coactivator binding has been reported so far. In this report, we present the design, synthesis and evaluation of non-peptide inhibitors of the interaction between activated VDR and a coactivator peptide fragment.

The X-ray structure of the complex consisting of 1,25$(OH)_2D_3$-bound VDR and a peptide fragment (residues 625–637: KNHPMLMNLLKDN-NH_2) of the coactivator DRIP 205 has been reported (Fig. 2a).[37] The LXXLL peptide adopts a short α-helical conformation, and binds in a surface groove formed by helices 3, 4, and 12 of VDR. The side chains of leucines 630, 633 and 634 of the LXXLL motif are buried within the pocket and surrounded by hydrophobic amino acids of VDR. In addition, the carboxylate oxygens of Glu 416 in VDR accept hydrogens from the main chain amides of Met 629 and Leu 630, and the primary amino group of Lys 242 in VDR donates a hydrogen to the main-chain carbonyl group of Leu 633. These hydrogen bonds form a 'charge clamp', as previously described for the interaction between other NRs and coactivators.[38]

To generate a non-peptide compound that inhibits the interaction between VDR and its coactivator, we employed structure-based drug design. We focused on five atoms in the pharmacophore of the peptide fragment as shown in Figure 2b because these atoms are expected to be spatially rigidly fixed due to α-helical conformation. We designed non-peptide templates that mimic the shape of the above-mentioned pharmacophore in the VDR-bound conformation of the coactivator. The benzodiazepine derivative **1** (Fig. 2c) was designed as a possible structure to match the pharmacophore, as shown in Figure 2d. Three branched alkyl groups in **1** were designed to mimic the leucine side chains in positions *i*, *i*+3 and *i*+4 of the α-helix. The carbonyl group in the diazepine moiety was anticipated to act as a part of the charge clamp by forming a hydrogen bond with the amino group of Lys 242. As the other part of the charge clamp, interacting with the carboxyl group of Glu 416, an anilino group was introduced to mimic the main chain of Leu 630. In addition, several side chains were introduced as candidate substitutes for the main chain of Met 629 that would interact with Glu 416.

Our plan for the synthesis of benzodiazepine **1** is outlined in Scheme 1. We envisioned a late-stage convergent installation of the charge clamp. The benzodiazepine **2**, in turn, was expected to be obtained by cyclization of **3** via Buchwald–Hartwig cross coupling reaction. Introduction of the *i*-butyl group was to be accomplished by Suzuki coupling reaction.

Nucleophilic substitution of commercially available **4** with 4-methoxybenzylamine followed by removal of the PMB group gave compound **6** (Scheme 2). Successive iodination[39] of **6** and reduction of the nitrile group afforded tetrasubstituted benzene **8**. After reductive amination of the primary amino group of **8**, amidation with *N*-Boc leucine furnished **10**. The regioselectivity of the reductive amination was confirmed with **7**, having diBoc protection (Scheme 3). DiBoc-protected **12** was converted to triBoc-protected **13** by the same method used to prepare **10** from **7**. Removal of the triBoc group of **13** followed by regioselective introduction of a Boc group at the primary amino group led to **10**. Next, two kinds of coupling reactions were investigated (Scheme 2). Suzuki coupling reaction[40] of **10** with boronate afforded **3** in 64% yield. Stille coupling reaction of **10** did not give a better result. After removal of the Boc group, cyclization by means of the intramolecular Buchwald–Hartwig coupling reaction[41] was studied (Table 1). When the reaction was carried at 85 °C according to the literature, the reaction proceeded in only 14% yield (entry 1). The highest yield (39%) of **2** was obtained when the reaction was carried at 110 °C (entry 2). Microwave-promoted reaction at 130 °C gave a complex mixture (entry 3). Hydrogenation of **2** gave **1a** possessing the saturated alkyl chain.

Introduction of the charge clamp moieties was achieved as shown in Scheme 4. Neither reductive amination of **1a** nor deprotection of the nosyl group introduced at the non-substituted anilino group of **1a** proceeded. Instead, alkylation of **1a** with alkyl bromide gave **1b** in 33% yield. After removal of the Boc group, acetylation of **1c** afforded **1d**. Guanidino derivative **1e** was synthesized from **1a** by the use of a pyrazole reagent.

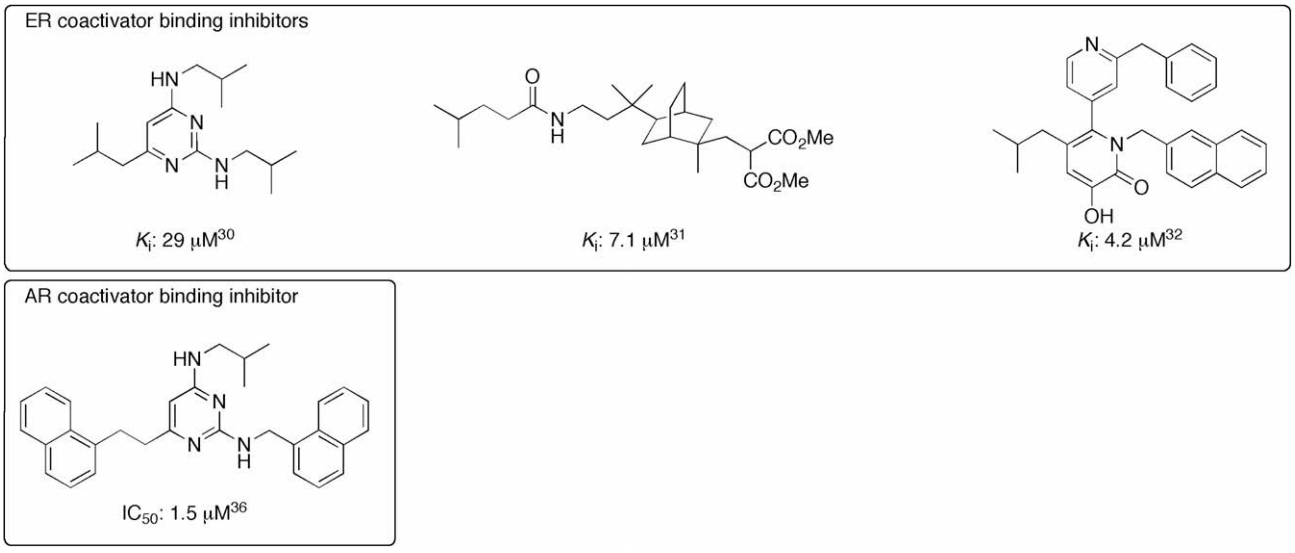


Figure 1. Reported inhibitors of binding between estrogen receptor (ER) or androgen receptor (AR) and their coactivators.

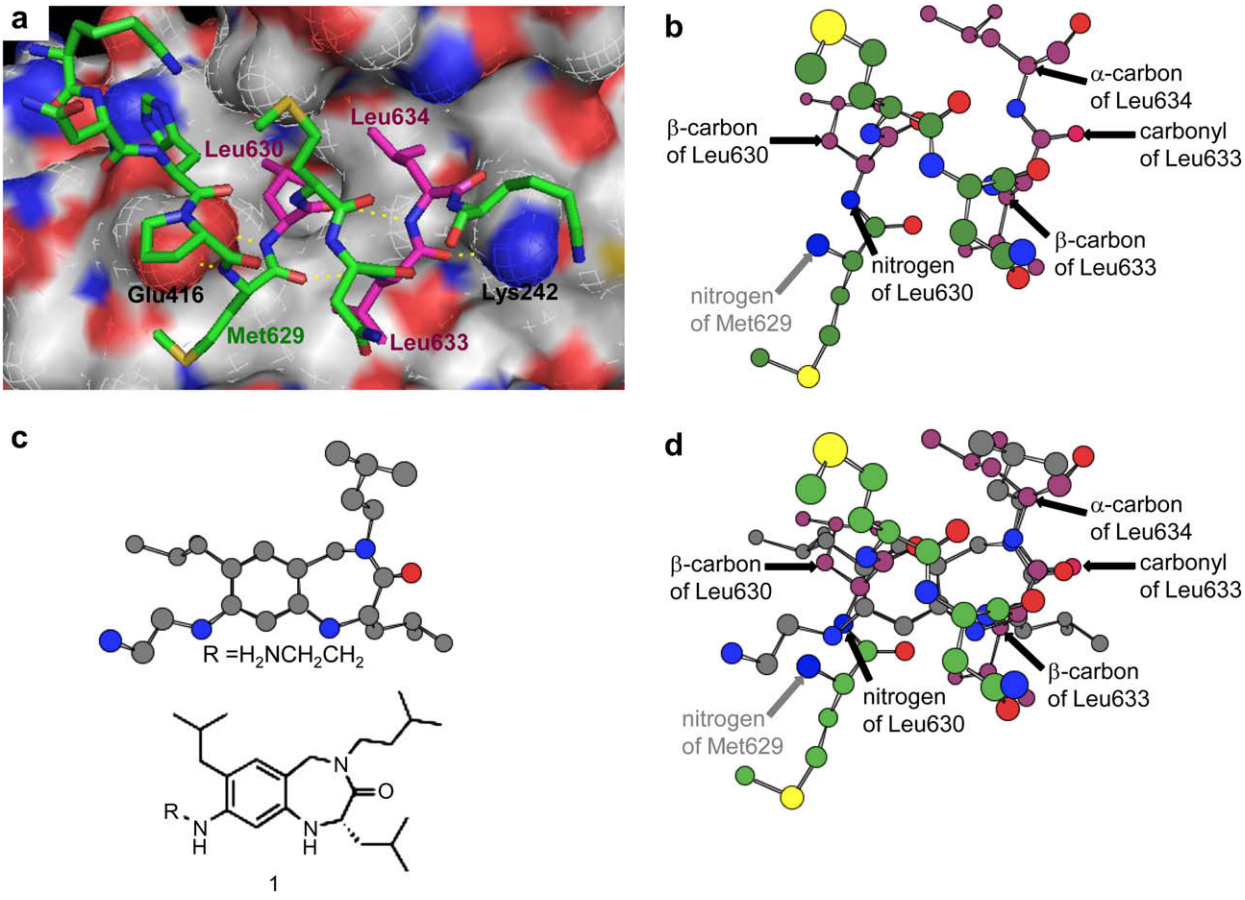
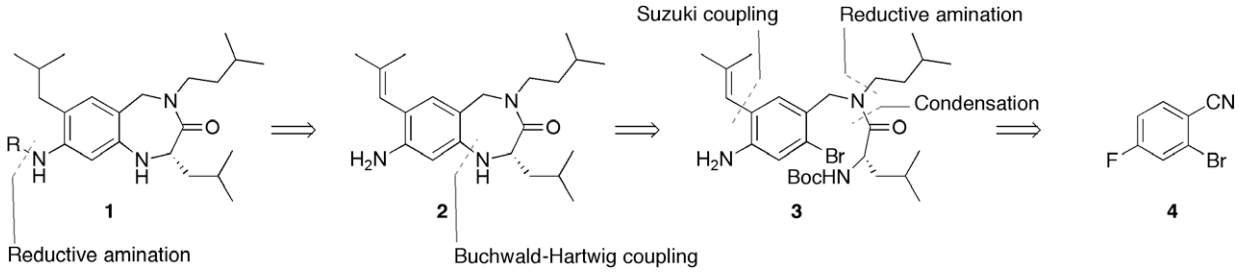


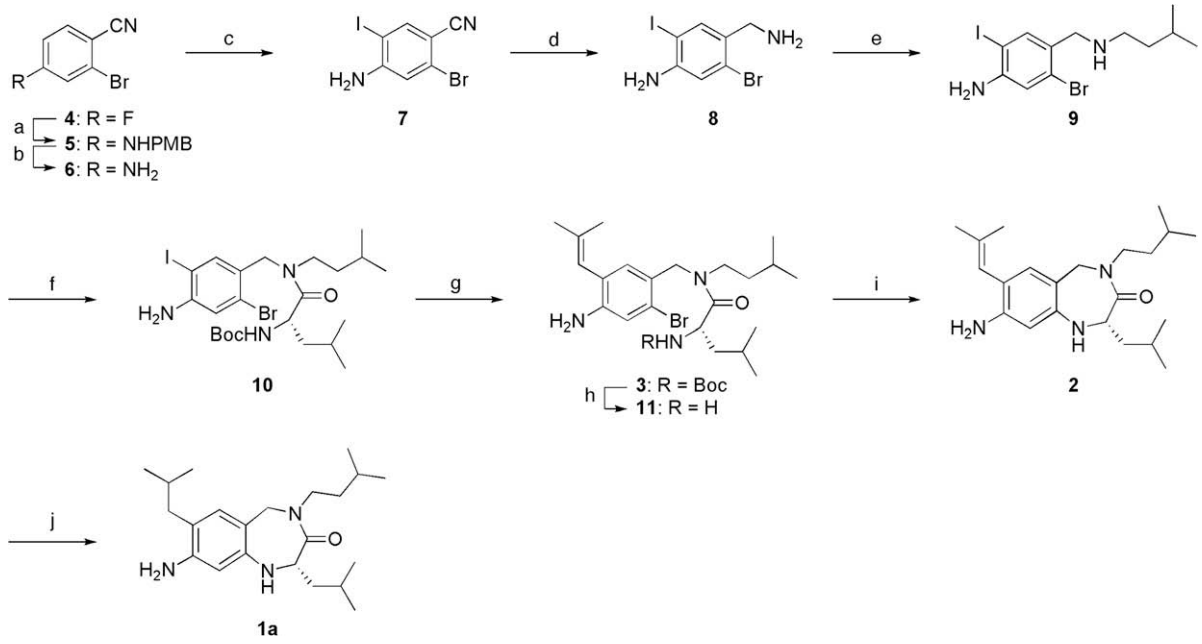
Figure 2. (a) Interaction between coactivator peptide fragment and VDR (PDB ID 1RK3). The VDR surface is shown in gray. The peptide is shown in green or magenta. Key leucines in the peptide are shown in magenta. The image was drawn with PYMOL. (b) Key coactivator peptide fragment (Met 629-Leu 634) and important atoms for the interaction. (c) Designed benzodiazepine **1**. (d) Superposition of (b) and (c).



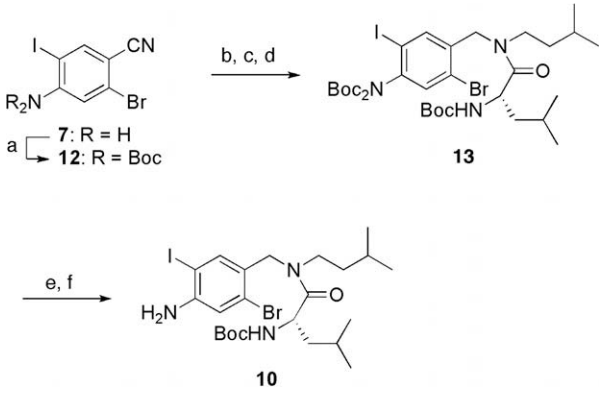
Scheme 1. Retrosynthetic analysis of benzodiazepine **1**.

We used a time-resolved fluorescence resonance energy transfer (TR-FRET) assay kit (Invitrogen) to evaluate the activity of synthesized compounds. Glutathione-*S*-transferase (GST)-tagged VDR-LBD (0.5 nM), terbium-labeled anti-GST antibody, a fluorescein-labeled fragment of coactivator peptide (100 nM), and $1,25(OH)_2D_3$ (10 nM) were mixed and incubated. The binding affinity between VDR and coactivator peptide is evaluated in terms of the TR-FRET ratio (520 nm:495 nm). In the presence of agonist ($1,25(OH)_2D_3$), the labeled coactivator peptide binds to VDR-LBD, allowing transfer of fluorescence resonance energy from Tb donor to the fluorescence acceptor. The binding affinity was maximum when the concentration of $1,25(OH)_2D_3$ was higher than 10 nM. Inhibition of binding between VDR and coactivator by synthesized compounds was assayed in terms of decrease of this FRET signal. The assay was performed in quintuplicate. After eliminating the highest and the lowest points, the average value of the remaining triplicate points was calculated. This assay was highly reproducible. A secosteroidal VDR antagonist DLAM-2P[17] was used as a positive control. Under the assay conditions used, DLAM-2P (10 μM) inhibited the binding by 87%. Synthesized compounds **1a** and **2** inhibited the FRET signal at 30 μM (Table 2). Among them, **2**[42] inhibited coactivator binding with an IC_{50} value of 20 μM. Compound **2,** possessing an isocrotyl group, showed stronger activity than saturated **1a**, which may indicate that the isocrotyl group fits better inside the hydrophobic pocket than does the isobutyl group.

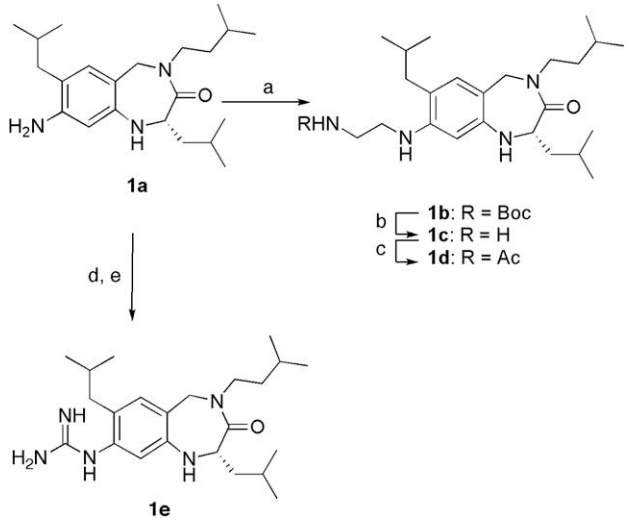
To investigate the cell-level VDR-inhibitory activity of the benzodiazepine series, we utilized a VDR-responsive reporter gene assay with CMX-GAL4N-hVDR LBD as the recombinant receptor gene, TK-MH100x4-LUC as the reporter gene, and the CMX β-galactosidase gene for normalization, as previously reported.[43] The cells were incubated with $1,25(OH)_2D_3$ (3 nM) in the presence or absence of a benzodiazepine or DLAM-2P. After incubation, cells were assayed for luciferase reporter gene and β-galactosidase activities. None of the compounds evaluated in our experiments reduced



Scheme 2. Reagents and conditions: (a) 4-methoxybenzylamine, 140 °C, 91%; (b) DDQ, DCM, H_2O, rt, 85%; (c) NIS, AcOH, rt, 82%; (d) BH_3, THF, 85 °C, 69%; (e) 3-methylbutanal, $NaBH(OAc)_3$, AcOH, DCM, 0 °C, 59%; (f) *N*-Boc-leucine, EDC, *i*-Pr_2EtN, HOBt, DCM, rt, 82%; (g) 2-methyl-1-propylboronic acid pinacol ester, $PdCl_2(dppf)$, K_3PO_4, DMF, 80 °C, 64%; (h) TFA, DCM, rt, 98%; (i) $Pd_2(dba)_3$, (*R*)-BINAP, Cs_2CO_3, toluene, 110 °C, 39%; (j) 10% Pd/C, H_2 (0.3 MPa), AcOEt, 50 °C, 100%.



Scheme 3. Reagents and conditions: (a) $(Boc)_2O$, DMAP, *i*-Pr_2EtN, THF, rt, 89%; (b) BH_3, THF, 100 °C, 55%; (c) 3-methylbutanal, $NaBH(OAc)_3$, AcOH, DCM, 0 °C, 76%; (d) *N*-Boc-leucine, EDC, *i*-Pr_2EtN, HOBt, DCM, rt, 71%; (e) TFA, DCM, rt, 24%; (f) $(Boc)_2O$, THF, 0 °C, 100%.



Scheme 4. Reagents and conditions: (a) $BocNHCH_2CH_2Br$, *n*-BuLi, THF, −78 °C, 33%; (b) HCl, 1,4-dioxane, rt, 42%; (c) AcOH, EDC, *i*-Pr_2EtN, HOBt, DCM, rt, 100%; (d) *N,N′*-diBoc-1*H*-pyrazole-1-carboxyamidine, *i*-Pr_2EtN, DMF, 65 °C, 29%; (e) HCl, 1,4-dioxane, 0 °C.

Table 1
Preparation of **2** by Buchwald–Hartwig coupling of **11**

Entry	Temperature (°C)	Time (h)	Yield (%)
1	85	8.5	14
2	110	11.5	39
3	130[a]	2	28

[a] A microwave reactor was used.

β-galactosidase activity in the concentration range investigated. Under the assay conditions used, the positive control DLAM-2P antagonized the $1,25(OH)_2D_3$-induced reporter gene luciferase activity with an IC_{50} value of 0.22 μM (Table 2). Compounds **1a** and **2** also caused a reduction in the $1,25(OH)_2D_3$-induced reporter gene luciferase activity. This result indicates that **1a** and **2** inhibit VDR-mediated transcriptional activation of responsive genes. The IC_{50} value of **2** in our assay system was calculated to be 17 μM. Next, we investigated whether benzodiazepine compounds compete with $1,25(OH)_2D_3$ for the vitamin D binding site. Addition of one hundred times higher concentration of $1,25(OH)_2D_3$ (300 nM) did not affect the observed reduction by **1a** and **2** of the $1,25(OH)_2D_3$-induced reporter gene luciferase activity (Fig. 3). On the other hand, the addition of a high concentration of $1,25(OH)_2D_3$ resulted in a dramatic decrease of the VDR-antagonistic efficacy of DLAM-2P, that is, the IC_{50} value of DLAM-2P was fifteen times higher (3.3 μM) in the presence of 300 nM $1,25(OH)_2D_3$ compared to that observed in the presence of 3 nM $1,25(OH)_2D_3$ (0.22 μM). These facts support the view that these benzodiazepine compounds are not competing with the ligand $1,25(OH)_2D_3$ as a conventional antagonist would, but rather they inhibit the binding between VDR and coactivator. Furthermore, selectivity with respect to other NRs was also investigated. ERα and ERβ reporter

Table 2
Inhibitory activity towards interaction between VDR and coactivator peptide fragment, and selectivity for other nuclear receptors

Compound	VDR			ERα	ERβ
	TR-FRET IC_{50} (μM)	Reporter gene IC_{50} (μM)	Reporter gene IC_{50} (μM)	Reporter gene IC_{50} (μM)	Reporter gene IC_{50} (μM)
Agonist	$1,25(OH)_2D_3$ (10 nM)	$1,25(OH)_2D_3$ (3 nM)	$1,25(OH)_2D_3$ (300 nM)	Estradiol (0.3 nM)	Estradiol (0.3 nM)
1a	>30 (48%)[a]	>30 (47%)[a]	30 (53%)[a]	>30 (8%)[a]	>30 (18%)[a]
2	20 (76%)[a]	17 (68%)[a]	23 (59%)[a]	>30 (8%)[a]	22 (56%)[a]
DLAM-2P	nt (87%)[b]	0.22 (98%)[b]	3.3 (66%)[b]	nt	nt

[a] Inhibition ratio at 30 μM.
[b] Inhibition ratio at 10 μM.

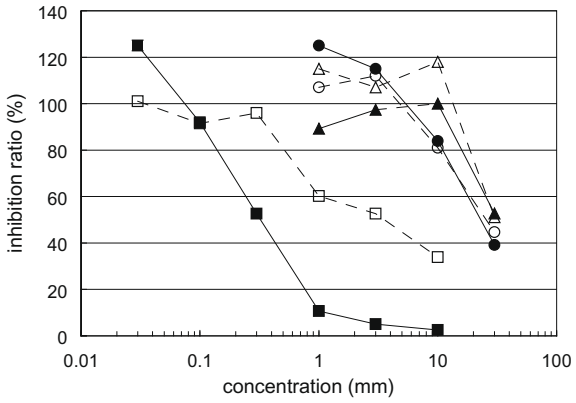


Figure 3. Dose–response of DLAM-2P (square), **1a** (triangle) and **2a** (circle) on VDR-responsive reporter gene activity. Closed symbols represent lower concentration of $1,25(OH)_2D_3$ (3 nM). Open symbols represent higher concentration of $1,25(OH)_2D_3$ (300 nM).

gene assay with CMX-GAL4N-hERα LBD or CMX-GAL4N-hERβ LBD as reporter genes, and estradiol (0.3 nM) as an ER agonist were used instead of CMX-GAL4N-hVDR LBD and $1,25(OH)_2D_3$. Compound **2** also inhibited ERβ reporter gene activity with an IC_{50} value of 22 μM, whereas **2** showed only a slight inhibitory effect on ERα reporter gene activity (Table 2). On the other hand, **1a** showed only slight inhibitory effects on ERα and ERβ reporter gene activities. Thus, VDR-coactivator binding inhibitors **1a** and **2** can be judged to be VDR-selective. Superposition of peptide fragments in coactivators of VDR, ERα[44] and ERβ[45] in the NR-bound conformation are shown in Figure 4. Peptide fragments of VDR and ERβ showed good overlap. On the other hand, the coactivator fragment of ERα did not match those of VDR and ERβ, especially at Leu 630 and Leu 634. This conformational difference is consistent with the selectivity of **1a** and **2**.

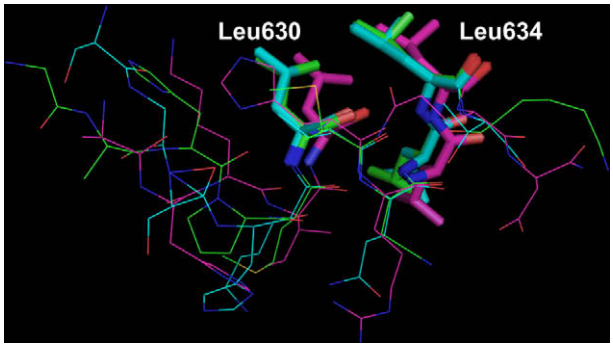


Figure 4. Superposition of peptide fragments of VDR-coactivator (green, PDB ID 1RJK), ERα coactivator (magenta, PDB ID 3ERD), and ERβ coactivator (cyan, PDB ID 1ZAF). Key leucines are shown as sticks. The image was drawn with PYMOL.

Table 3
Inhibitory activity of **1b**–**e** on the interaction between VDR and coactivator peptide fragment

Compound	VDR TR-FRET IC_{50} (μM)
1b	13
1c	28
1d	>30 (43%)[a]
1e	>30 (46%)[a]

[a] Inhibition ratio at 30 μM.

Because proof of principle for inhibition of the interaction of VDR with coactivators had been achieved, we next introduced side chains in an attempt to improve the activity. All the synthesized benzodiazepine molecules inhibited the FRET signal at 30 μM (Table 3). Among them, **1b** and **1c** inhibited coactivator binding with IC_{50} values of 13 and 28 μM, respectively. Compounds **1c**–**e** showed weaker activity, indicating that the introduced side chains were unable to interact with Glu 416. On the other hand, **1b**[46] possessing a bulky side chain exhibited the strongest activity, implying that the side chain of **1b** might mimic the side chain of Met 629, thereby affording an additional hydrophobic interaction with VDR.

In conclusion, benzodiazepine molecules were rationally designed as non-peptide LXXLL mimetics based on the VDR-coactivator interaction. TR-FRET assay showed that **2** inhibits the interaction between VDR and a coactivator peptide fragment with an IC_{50} value of 20 μM. Compound **2** also inhibited VDR-mediated transcription with almost the same IC_{50} value (17 μM) in reporter gene assay. Inhibition of VDR-mediated transcription by **2** was independent of the concentration of co-existing agonist. Furthermore, VDR-coactivator interaction inhibitor **2** did not inhibit ERα-mediated transcription. Although these inhibitory activities were not highly potent, this is the first report of non-peptide inhibitors of the binding between VDR and its coactivators, and of non-secosteroidal inhibitors of VDR-mediated transcription to our knowledge. Structural development of the benzodiazepine series and further studies on selectivity with respect to other nuclear receptors are in progress.

Acknowledgments

The work described in this paper was partially supported by Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Japan Society for the Promotion of Science. We thank Dr. Motomu Kanai for carrying out the microwave-promoted reaction.

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42. Compound **2**: ^{1}H NMR (500 MHz, $CDCl_3$–D_2O) δ 6.56 (s, 1H), 5.94 (s, 1H), 5.86 (s, 1H), 5.26 (d, J = 16.4 Hz, 1H), 4.53 (t, J = 6.7 Hz, 1H), 3.65 (d, J = 16.4 Hz, 1H), 3.58–3.52 (m, 1H), 3.43–3.38 (m, 1H), 1.87 (s, 3H), 1.83–1.75 (m, 2H) 1.71 (s, 3H), 1.51–1.35 (m, 4H), 0.96 (d, J = 6.7 Hz, 3H), 0.95 (d, J = 6.1 Hz, 3H), 0.88 (d, J = 7.9 Hz, 3H), 0.83 (d, J = 6.7 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ; 170.6, 144.9, 144.7, 136.4, 131.1, 120.3, 114.8, 110.7, 102.9, 52.1, 51.4, 45.7, 40.4, 37.2, 25.9, 25.9, 24.7, 23.0, 22.5 (3C), 19.4; MS(FAB) m/z 357 $(M)^+$, 358 $(M+H)^+$.
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46. Compound **1b**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.52 (s, 1H), 5.74 (s, 1H), 5.26 (d, J = 15.9 Hz, 1H), 4.98–4.87 (m, 2H), 4.80–4.72 (m, 1H), 4.56–4.49 (m, 1H), 3.64 (d, J = 16.5 Hz, 1H), 3.54 (t, J = 4.9 Hz, 2H), 3.53–3.47 (m, 1H), 3.46 (t, J = 4.9 Hz, 2H), 3.20–3.15 (m, 2H), 2.22 (d, J = 6.1 Hz, 2H), 1.90–1.84 (m, 1H), 1.83–1.76 (m, 2H), 1.52–1.48 (m, 3H), 1.45 (s, 9H), 1.40–1.35 (m, 3H), 0.96 (d, J = 6.8 Hz, 3H), 0.95 (d, J = 6.1 Hz, 3H), 0.92 (d, J = 6.7 Hz, 3H), 0.92 (d, J = 6.8 Hz, 3H), 0.88 (d, J = 6.1 Hz, 3H), 0.83 (d, J = 6.7 Hz, 3H); MS (FAB) m/z 502 (M^+), 503 $(M+H)^+$.