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Development of Novel Vitamin D Receptor−Coactivator Inhibitors

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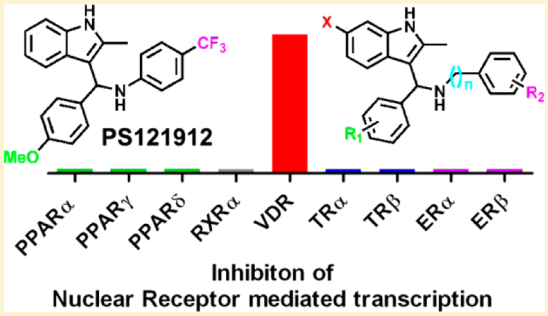
Supporting Information

ABSTRACT: Nuclear receptor coregulators are master regulators of transcription and selectively interact with the vitamin D receptor (VDR) to modulate cell differentiation, cell proliferation, and calcium homeostasis. Herein, we report the syntheses and evaluation of highly potent and selective VDR−coactivator inhibitors based on a recently identified 3-indolylmethanamine scaffold. The most active compound, PS121912, selectively inhibited VDR-mediated transcription among eight other nuclear receptors tested. PS121912 is also selectively disrupting the binding between VDR and the third nuclear receptor interaction domain of the coactivator SRC2. Genetic studies revealed that PS121912 behaves like a VDR antagonist by repressing 1,25-$(OH)_2D_3$ activated gene transcription. In addition, PS121912 induced apoptosis in HL-60.



KEYWORDS: *Vitamin D receptor, VDR, steroid receptor coactivator, fluorescence polarization, high throughput screening, 3-indolyl-methanamines, CYP24A1, CAMP, apoptosis, HL60*

The vitamin D receptor (VDR) is a ligand-inducible transcription factor that belongs to the superfamily of nuclear receptors (NR).[1] Like most NRs, VDR has a modular structure consisting of a DNA binding domain and a ligand binding domain (LBD) connected by a flexible hinge region. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), a hormonally active form of vitamin D, is the endogenous high affinity ligand for VDR.[2] Traditionally, 1,25-$(OH)_2D_3$ is known for its role in cell differentiation and calcium homeostasis, but lately it has been reported to be involved in inflammation, immune response, and cell proliferation.[3]

Human clinical studies with 1,25$(OH)_2D_3$ are dose-limited because of the reported induction of hypercalcemia and hypercalciurea.[4,5] This dysregulation can cause psychosis, bone pain, calcification of soft tissue, coronary artery disease, and, in severe cases, coma and cardiac arrest. During the last decades, hundreds of VDR agonists have been synthesized to develop VDR ligands with lower calcemic activity. However, two synthetic VDR ligands that were developed to systemically treat cancer were not active or induced hypercalcemia in clinical trials.[6,7] In addition, a smaller number of VDR antagonist were developed, which include the irreversible antagonist TEI-9647[8] and those bearing bulky side chains such as 25-carboxylic esters (ZK168218 and ZK159222),[9] 26-adamantly substituted antagonists (ADTT and analogues),[10] and 22-butyl-branched compounds that ultimately destabilized the active form of VDR.[11] Interestingly, none of these antagonists have been further developed as therapeutics.

Recently, coregulator proteins have been identified as transcriptional master regulators.[12] In its unligated state, VDR is bound with corepressors. Upon binding with 1,25-$(OH)_2D_3$, VDR undergoes a conformational change that disrupts corepressor binding and enables the interaction between VDR and coactivators. The activated form of VDR interacts with retinoid X receptor (RxR) to form a heterodimer that binds to vitamin D response elements on the DNA leading to transcription of associated genes. VDR is known to interact with 2700 human DNA sites and effects the expression of 229 gene products.[13]

A new approach to modulate VDR function without causing hypercalcemia but to induce cell proliferation and differentiation is the design of small molecules that target the interactions between VDR and coregulators. In general, the approach of inhibiting protein−protein interactions is challenging in terms of potency and specificity. Further adding to the complexity is the fact that the family of more than 300 coregulators interact with different NR.[14] Thus, selectivity with respect to different NRs and inhibition of specific NR−

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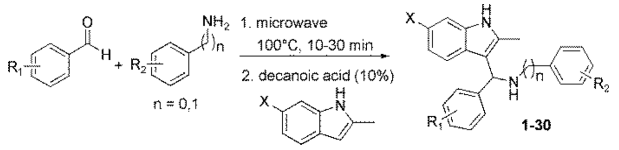
Scheme 1. Improved Synthesis of 3-Indolylmethanamines



coregulator interactions is essential for novel promising NR–coregulator inhibitors.

Various small molecule NR–coactivator inhibitors have been discovered for thyroid receptor (TR), pregane X receptor (PxR), estrogen receptor (ER), and the androgen receptor (AR).[15] Recently, the first reversible VDR–coactivator inhibitors were developed to disrupt the VDR-mediated gene transcription, but these compounds lack the important selectivity among other NRs.[16,17] However, drawing from a pool of hundreds of thousands of molecules, high throughput screening (HTS) is a superior method to identify ideal candidates that not only possess selectivity for VDR but also for the various VDR–coregulator interactions.

The first HTS method to quantify the interaction between VDR and coregulator peptides was introduced by Arai et al. using fluorescence intensity.[18] Six compounds were investigated in this study. Recently, we described a HTS campaign in collaboration with NIH investigating 390 000 molecules that led to the identification of GW0742 as a competitive VDR antagonist.[19] Furthermore, we reported a second HTS campaign with 275 000 molecules that resulted in the development of an irreversible VDR–coactivator binding inhibitor.[20] Herein, we report our recent successes to improve the potency and selectivity of these VDR–coactivator binding inhibitor. Importantly, we demonstrated that these unique compounds modulated gene transcription and cell proliferation.

A diversified library of 3-indolylmethanamines was generated by using one-pot modified microwave-based Aza–Friedel–Craft reaction (Scheme 1).

The first step involves the combination of an amine and an aromatic aldehyde to form an imine intermediate at 100 °C within 10 to 30 min using microwave irradiation. To that solution, indole and a catalytic amount of decanoic acid was added to afford the desired products at room temperature, which are summarized in Figure 1. The purified compounds were analyzed using a battery of biophysical and biochemical assays (Table 1).

The biophysical properties determined include small molecule solubility and permeability. The solubility of synthesized 3-indolylmethanamines in PBS buffer (pH 7.4) with 5% DMSO ranged between 150 and 3 μM. The compounds substituted with polar heterocyclic side chains showed excellent solubility (>100 μM). The small molecule permeability was determined using a parallel artificial membrane permeation assay (PAMPA) employing a hexadecane membrane. In comparison to the used standards (ranitidine = −8.02 ± 0.074 cm/s (low permeability), carbamazepine = −6.81 ± 0.0011 cm/s (medium permeability), and verapamil = −5.93 ± 0.015 cm/s (high permeability), the majority of 3-indolylmethanamines exhibited medium to high permeability (Table 1).

A fluorescence polarization (FP) assay was used to determine the ability of synthesized molecules to inhibit the interactions between VDR-LBD and Alexa Fluor 647 labeled coactivator peptide SRC2–3. The compounds were analyzed in a dose-dependent manner, and potencies are reported as IC_{50} values. In order to assess the ability of 3-indolylmethanamines to

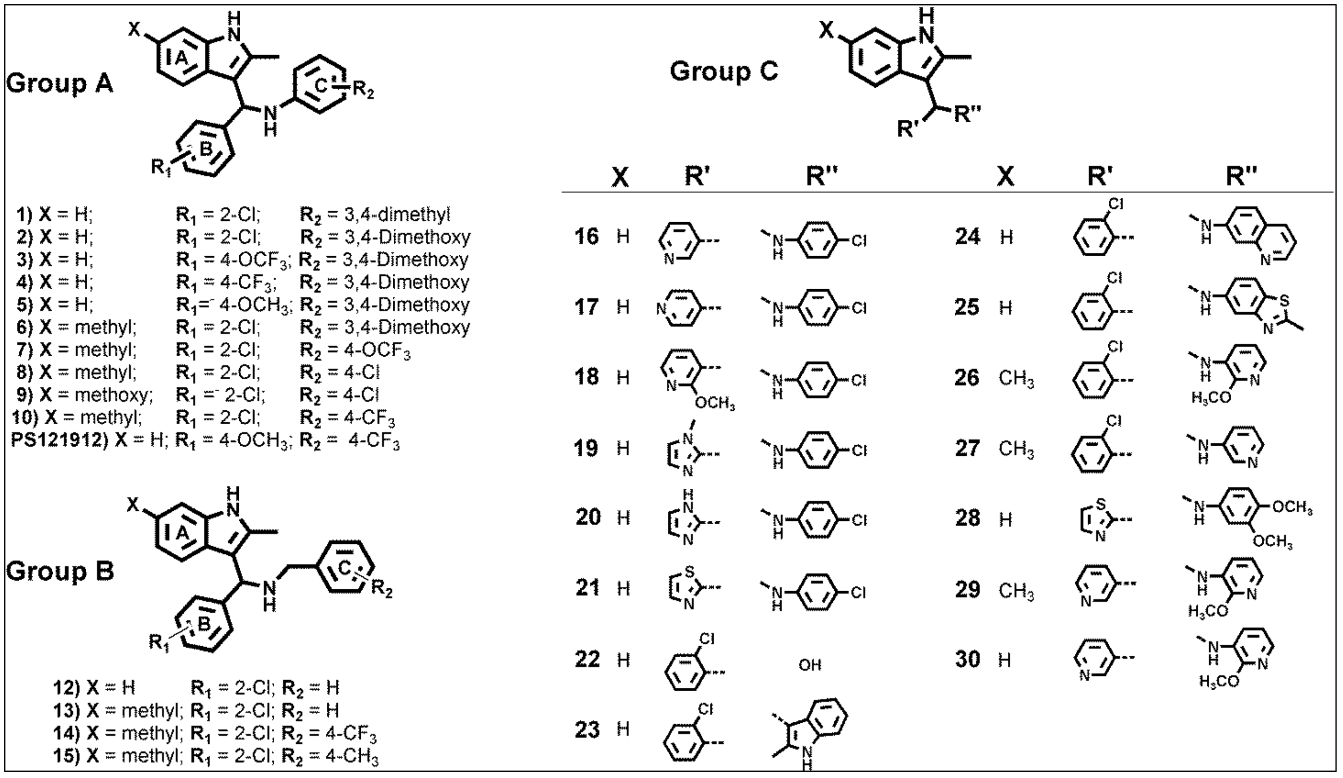


Figure 1. Structures of synthesized 3-indolylmethanamines.

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Table 1. Summary of Biophysical and Biochemical Properties of 3-Indolylmethanamines

compd	solubility (μM)[a]	permeability[b] log (Pe) (cm/s)	VDR-SRC2−3 IC_{50} (μM)[c]	VDR-mediated transcription IC_{50} (μM)[d]	toxicity LC_{50} (μM)[e]
1	3.6	7.36 ± 0.10	12.8 ± 0.8	n.o.	36.4 ± 7.4
2	57.9	7.03 ± 0.07	7.2 ± 0.4	3.0 ± 0.7	>50
3	96.5	6.26 ± 0.02	22.8 ± 2.2	2.2 ± 0.5	15.3 ± 1.7
4	91.5	6.64 ± 0.02	21.8 ± 1.7	4.4 ± 2.1	14.1 ± 1.6
5	94.0	6.16 ± 0.02	11.3 ± 0.5	3.2 ± 0.7	41.7 ± 13.3
6	48.1	5.86 ± 0.03	n.o.	9.3 ± 3.1	>100
7	25.2	6.26 ± 0.01	17.2 ± 4.8	5.0 ± 3.4	>75
8	8.5	7.42 ± 0.52	15.6 ± 1.4	5.2 ± 2.7	>50
9	19.8	7.68 ± 0.31	63.1 ± 13.6	7.3 ± 2.9	>100
10	29.4	n.d.	n.o.	5.8 ± 3.1	>75
PS121912	68.9	6.36 ± 0.02	12.4 ± 0.7 (8.1 ± 2.3)[f]	0.59 ± 0.1	27.3 ± 2.7
12	60.4	6.21 ± 0.01	7.2 ± 0.4	5.4 ± 3.5	42.7 ± 2.7
13	45.5	5.60 ± 0.19	59.9 ± 4.5	5.8 ± 2.1	>75
14	18.2	6.67 ± 0.04	35.2 ± 12.5	9.1 ± 2.2	53.7 ± 16.8
15	20.1	6.41 ± 0.01	9.5 ± 0.4	3.4 ± 0.6	10.8 ± 1.6
16	123.3	6.47 ± 0.11	>75	3.2 ± 1.4	>100
17	128.3	6.50 ± 0.14	51.5 ± 10.4	3.7 ± 1.8	>100
18	52.8	7.57 ± 0.32	14.2 ± 1.4	14.1 ± 6.6	>100
19	3.6	n.d.	66.8 ± 10.3	n.o.	>100
20	176.7	6.71 ± 0.01	16.7 ± 0.8	6.1 ± 2.5	>75
21	97.9	6.14 ± 0.13	11.9 ± 0.7	3.8 ± 2.1	>75
22	99.3	6.92 ± 0.16	n.o.	n.o.	>100
23	114.4	n.d.	>75	n.o.	>75
24	3.0	n.d.	n.o.	>25	>100
25	95.3	6.68 ± 0.11	15.8 ± 1.2	>25	>75
26	130.1	5.87 ± 0.01	101.4 ± 15.2	12.2 ± 3.3	>75
27	120.3	6.09 ± 0.01	29.3 ± 5.7	6.7 ± 2.5	>100
28	101.8	6.14 ± 0.04	17.3 ± 0.7	8.5 ± 2.7	>50
29	150.2	5.78 ± 0.01	32.3 ± 6.4	5.4 ± 2.1	18.6 ± 2.5
30	144.8	5.88 ± 0.02	n.o.	n.d.	>75

[a]Solubility was determined in phosphate-buffered saline at pH 7.4. [b]Permeability was measured using the parallel artificial membrane permeation assay (PAMPA) at neutral pH (pH = 7.4). [c]A fluorescence polarization competition assay was carried out using VDR-LBD (1 μM), Alexa Fluor-labeled peptide SRC2−3 (7 nM), VDR-agonist LG190178 (5 μM), and serial diluted small molecules. IC_{50} values were obtained by fitting data obtained after 2 h to the following equation: Y = bottom + (top − bottom)/(1 + $10^{((\log IC_{50} - X)\text{hillslope})}$) using three independent experiments in quadruplet. [d]Transcription assay: HEK293T cells were transfected with CMV-VDR and a CYP24A1 promoter driven luciferase expression vector in the presence of $1,25(OH)_2D_3$. [e]Toxicity was determined under the conditions of the transcription assay using CellTiter-Glo. [f]Two-hydrid assay: HEK293T cells were transfected with a VP16-VDR-LBD, SRC1-GAL4, and luciferase reporter plasmid vector in the presence of $1,25(OH)_2D_3$.[21] n.d. = not determined; n.o. = not observed.

inhibit the VDR−coactivator interaction in cells, a VDR two-hybrid assay and a VDR-mediated transcription assay was used. The toxicity of compounds under the conditions of the transcription assay was determined with CellTiter-Glo (Promega).

All 3-indolylmethanamines in group A (Table 1, compounds 1−10 and PS121912) exhibited cellular activities in the low micromolar to nanomolar range. The compound activities measured with the biochemical FP assay are generally higher probably due to compound off-targets effects. The compound toxicities are ranging between 14.1 and >100 μM. The compound PS121912 exhibited the highest activity in the VDR-mediated transcription assay (IC_{50} = 590 ± 100 nM) and largest therapeutic index.

For compounds in group B, bearing benzylamine substitutents, low micromolar activities were determined for the transcriptional inhibition of VDR. The activities for the FP assay ranged between 7.2 to 59.9 μM. Importantly, 3-indolylmethanamines are irreversible inhibitors acting through the formation of an azafulvenium salt that react with nucleophilies like mercaptoethanol (see Supporting Information). Thus the activity of these inhibitors depends on the incubation time, the environment, and the electronic substituent effects.[20] Compound 15 was the most toxic compound within the library of 3-indolylmethanamines with a LD_{50} value of 10.8 ± 1.6 μM.

For compounds in group C, various heterocyclic substituents were introduced. Interestingly, the majority of these 3-indolylmethanamine were not toxic but very active inhibitors of VDR-mediated transcription. Compound 16 exhibited the largest therapeutic index of more than 31 in group C, but it was still inferior to compound PS121912 with a therapeutic index of 46. The substitution of the secondary nitrogen by oxygen or carbon prevented the generation of a reactive electrophilic compound and thus resulted in inactive compounds 22 and 23.

The NR-selectivity of the most potent compound, PS121912, was determined by measuring the inhibition of transcription for a panel of nine different NRs. These include the peroxisome proliferator-activated receptors α, γ, and δ, the retinoic acid receptor α, the thyroid receptors α and β, and the estrogen receptors α and β. The results are summarized in Table 2.

The 3-indolylmethanamine PS121912 selectively inhibited the transcription of VDR at nanomolar concentrations, whereas for all other NRs we determined IC_{50} values ranging between

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Table 2. Inhibition of NR-Mediated Transcription in the Presence of Compound PS121912[b]

compd	nuclear receptor	IC_{50} (μM)[a]
1	VDR	0.59 ± 0.1[b]
2	PPAR-α	20.8 ± 1.1[c]
3	PPAR-γ	>25[d]
4	PPAR-δ	>30[e]
5	RxR-α	>25[f]
6	TR-α	>30[g]
7	TR-β	24.1 ± 1.1[g]
8	ER-α	26.2 ± 3.3[h]
9	ER-β	>25[h]

[a]Three independent experiments were conducted in quadruplicate, and data was analyzed using nonlinear regression with variable slope (GraphPrism). [b]1,25$(OH)_2D_3$ (10 nM). [c]GW7647 (30 nM). [d]Rosiglitazone (300 nM). [e]GW0742 (50 nM). [f]Bexarotene (200 nM). [g]T3 (10 nM). [h]Estradiol (10 nM).

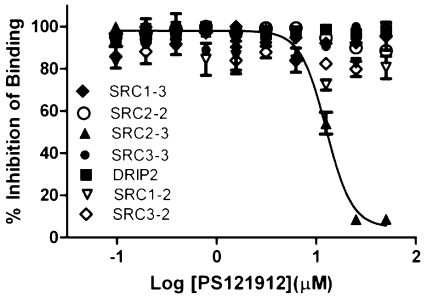

Figure 2. Selectivity studies for VDR−coactivator interactions inhibition in the presence of PS121912 using FP assay. VDR-LBD (1−10 μM), VDR agonist LG190178 (5 μM), and different coactivator peptides (7 nM) were incubated for 3 h in the presence of different concentrations of compound PS121912.

20 and 26 μM. It is important to point out that the LD_{50} value of PS121912 in HEK293T cells was 27.3 μM; thus, the reduced luciferase activity measured at concentrations above 20 μM is probably due to cell death and not caused by inhibition of NR-mediated transcription. Therefore, the NR selectivity of PS121912 is at least 35-fold.

The selectivity of PS121912 to inhibit the interaction between VDR and coactivator SRC1,[22] SRC2,[23] SRC3,[24] or DRIP205[25] was assessed using FP assays employing different NR interaction domains (NIDs) of these coactivators (Figure 2).

The quantification of interactions between VDR and different coactivator peptides was reported recently.[26] Among the interactions tested, only the interactions between VDR and coactivator peptide SRC2−3 was fully inhibited by compound PS121912 with an IC_{50} value of 12.4 ± 0.7 μM (Table 1). The NIDs of SRC1, SRC3, and DRIP2, which exhibit a similarly strong interaction with VDR, were not inhibited by PS121912. A very weak partial inhibition for the VDR−SRC2−2 interaction was observed in the presence of PS121912 leaving 88% of the VDR−coactivator binding intact.

Furthermore, the expression levels of VDR target genes CYP24A1[27] and CAMP[28] were determined in HL-60 cells treated with 7.5 μM of compound PS121912 in the presence and absence of 20 nM 1,25-$(OH)_2D_3$ for 18 h. The results are summarized in Figure 3.

A strong induction of CYP24A1 and CAMP by 1,25-$(OH)_2D_3$ was observed. Cells treated 1,25-$(OH)_2D_3$ and

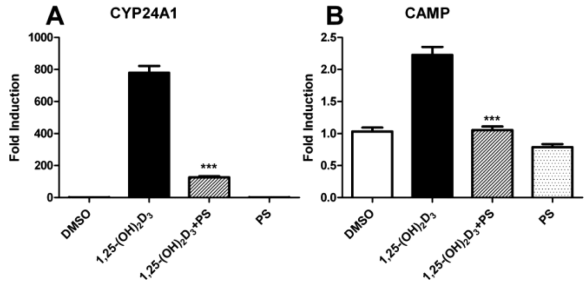

Figure 3. Gene regulation by PS121912 (7.5 μM) in HL-60 cells after 18 h in the presence and absence of 1,25-$(OH)_2D_3$ (20 nM). Standard errors of mean were calculated from two biological independent experiments performed in triplicate. Stars represent $P < 0.001$ (***) (Student's t test).

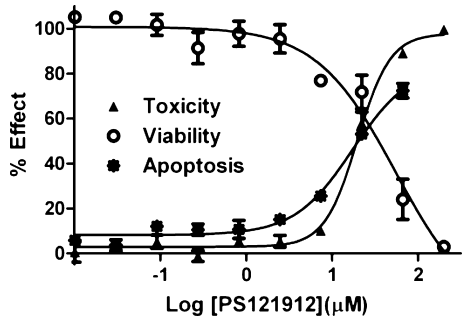

Figure 4. HL-60 viability, toxicity, and apoptosis assay after 18 h in the presence of PS121912.

compound PS121912 exhibited a loss of induction of transcription, whereas cells treated with only PS121912 showed comparable expression to that of vehicle treated HL-60 cells. The transcriptional inhibition of the CYP24A1 gene product 24-hydroxylase by PS121912 is important because it has been shown that deactivation of 24-hydroxylase, using vitamin D analogues[29] or general P450 enzyme inhibitors[30] promotes antiproliferation and differentiation of cancer cells.

Therefore, viable, apoptotic, and dead HL-60 cells were determined in the presence of different concentrations of PS121912 as depicted in Figure 4.

Three different assays were used: (a) CellTiter-Glo that quantifies the amount of ATP (toxicity), (b) CellTiter-Fluor that quantifies the amount of active cellular proteases (viability), and (c) Caspase-Glo 3/7 that quantifies the amount of active cellular caspase 3 and 7 (apoptosis). As expected, the amount of intact and dead HL-60 cells at the same concentration of PS121912 was inversely proportional with EC_{50} values of around 20 μM. Importantly, a dose-dependent increase of caspase 3 and 7 activity was observed in the presence of PS121912. The increase of the apoptotic enzymes was proportional to the amount of dead cells, confirming that programmed cell death and not necrosis was the mode of action of PS121912 at higher concentrations.

■ ASSOCIATED CONTENT

Supporting Information

Detailed procedures and characterization of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

NR, nuclear receptors; VDR, vitamin D receptor; LBD, ligand binding domain; SRC, steroid receptor coactivator; DMSO, dimethylsulfoxide; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; RxR, retinoid X receptor; TR, thyroid receptor; PxR, pregame X receptor; ER, estrogen receptor; AR, androgen receptor; HTS, high throughput screening; PAMPA parallel artificial membrane permeation assay; CYP24A1, cytochrome P450_24A1; CAMP, cathelicidin antimicrobial peptide gene

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