Fluorescence polarization (FP) assay for identification of vitamin D receptor (VDR) ligands

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Introduction

The vitamin D receptor (VDR) is a ligand-dependent transcription factor in the nuclear receptor gene superfamily. VDR not only plays a central role in maintaining the structural integrity of bones, but also modulates the immune response and cell proliferation and differentiation. In addition to the utility of vitamin D analogs in the treatment of bone disorders and psoriasis, there is continuing interest in the discovery of VDR-active drugs for the treatment of immune disorders and malignant tumors. In particular, VDR modulators may be useful in the treatment of autoimmune conditions such as multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel diseases (IBDs), type I diabetes, systemic lupus erythematosus (SLE), and organ and tissue transplant rejection. VDR modulators are also of interest in leukemia, squamous cell carcinoma, Kaposi's sarcoma, and cancers of the breast, prostate, and colon.

Progress in this area has been hampered by the absence of a suitable high-throughput assay for measurement of ligand affinity for VDR. To overcome this limitation, we have developed both fluorescence polarization (FP) and time-resolved fluorescence resonance energy transfer (TR-FRET) assays based on the ability of competitive ligands to displace a novel fluorescent ligand, Fluormone™ VDR Red, from purified VDR. The robustness of the FP assay (Z'-factor values ≥0.7 in 384-well format) demonstrates its suitability for high-throughput screening of potential VDR ligands.

Materials and Methods

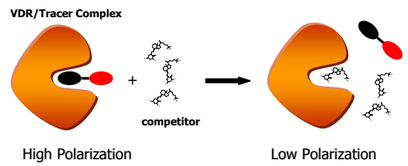
VDR ligands – 1α-dihydroxyvitamin D_3 (calcitriol) and calcipotriol were purchased from Exim Pharm International, and 1α-hydroxyvitamin D_3 (1α-OH D_3), 25-hydroxyvitamin D_3 (25-OH D_3), and vitamin D_3 were from Sigma-Aldrich.

Fluorescence polarization VDR Red competition assay – Full-length human VDR, Fluormone™ VDR Red, and VDR Red Screening Buffer were from Invitrogen. Assays were performed in 384-well black polypropylene plates (MatriCal, Inc.) using a total volume of 40 µL/well. Receptor/tracer complex was added to ligand or DMSO solvent control to final concentrations of 0.7 nM VDR and 1 nM Fluormone VDR Red. Fluorescence polarization was measured on an Ultra384 microplate reader (Tecan) using a 535 nm excitation filter (25 nm bandwidth) and 590 nm emission filter (20 nm bandwidth).

Time-resolved fluorescence resonance energy transfer (TR-FRET) VDR Red competition assay – VDR ligand-binding domain (VDR-LBD) tagged with GST at the N-terminus and terbium-labeled anti-GST antibody (Tb-anti-GST) were from Invitrogen. Assays were performed in 384-well black polypropylene plates (MatriCal) using a total volume of 40 µL/well. Receptor/tracer/antibody complex was added to ligand or DMSO solvent control to final concentrations of 5 nM Tb-anti-GST and the receptor/tracer concentrations indicated in each figure. TR-FRET was measured on an Ultra384 microplate reader (Tecan) using a 340 nm excitation filter (30 nm bandwidth), tracer emission filter 570 nm (10 nM bandwidth), and terbium emission filter 546 nm (10 nM bandwidth). A 100 µs post-excitation delay and 200 µs signal integration time were used.

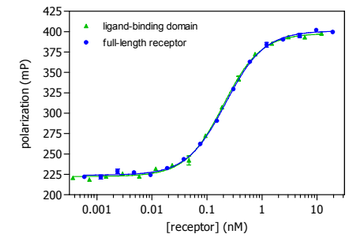
Hydroxylapatite (HAP) ^{3}H-calcitriol competition assay – Full-length human VDR (0.7 nM) and 1 nM ^{3}H-calcitriol (Perkin Elmer) were incubated at room temperature for 3 hours in the presence of varying concentrations of ligand or DMSO control in VDR Red Screening Buffer. A 50% HAP slurry was then added to bind the receptor, and wash steps were conducted to remove unbound ^{3}H-calcitriol. Radioactivity of the HAP pellet containing the VDR/^{3}H-calcitriol complexes was then measured using a Beckman Coulter LS6500 Scintillation Counter.

Figure 1 – FP Assay Principle



When VDR binds the fluorescent Fluormone™ VDR Red (also referred to as "tracer", indicated with red tag), the resulting complex yields a high fluorescence polarization value (mP). VDR ligands can be identified by their ability to displace the tracer from the complex, resulting in a lower fluorescence polarization value. This shift in polarization enables determination of the relative binding affinity of a test compound for VDR.

Figure 2 – Fluormone VDR Red Binding to VDR



Full-length receptor or VDR-LBD was titrated in the presence of 1 nM Fluormone™ VDR Red to measure binding affinity. The apparent K_d value for both the full-length and tuncated VDR protein was determined to be ~0.2 nM.

Figure 3 – Simple "Mix and Read" Protocol

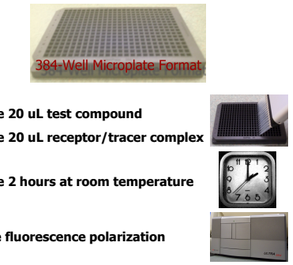
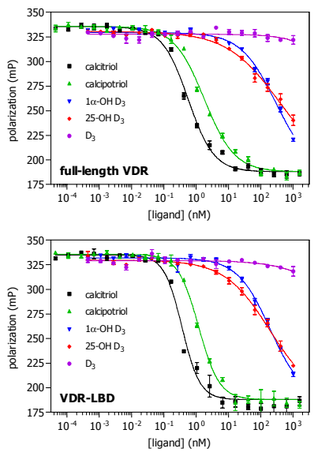


Table 1 – VDR Red FP Assay Robustness

Incubation Time	ΔmP	Z'-Factor (n=32)	Calcitriol IC_{50} (nM)
2 hrs	139 ± 3	0.74 ± 0.02	2.3 ± 0.3
4 hrs	136 ± 2	0.78 ± 0.01	1.9 ± 0.2
6 hrs	135 ± 2	0.75 ± 0.03	1.9 ± 0.1

The VDR Red Assay reaches equilibrium at room temperature after 2 hours and has a 4-hour read window where ΔmP and IC_{50} values are stable and excellent Z'-factor values are achieved. ΔmP is defined as the difference in polarization values between receptor-bound tracer and tracer displaced by 1 µM calcitriol (1% DMSO in all wells). Data represents the average ± 1 standard deviation from 3 separate experiments.

Figure 4 – Displacement of Fluormone VDR Red by VDR Ligands



1 nM Fluormone™ VDR Red and either 0.7 nM full-length VDR or VDR-LBD were incubated for 4 hours at room temperature in the presence of varying amounts of several vitamin D-based molecules (2.5% DMSO). Data points represent the average and standard error of 3 replicate wells. IC_{50} values are summarized in Table 2.

Figure 5 – Structures of Secosteroidal VDR Ligands

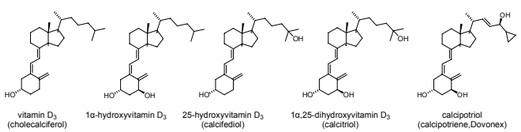
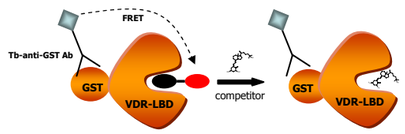


Table 2 – VDR Assay IC_{50} Values (nM)

Ligand	Full-length ^{3}H-calcitriol	Full-length FP	LBD FP
calcitriol	2.0	0.6	0.4
calcipotriol	7.3	1.8	1.2
1α-OH D_3	930	290	210
25-OH D_3	1420	370	210
D_3	ND	ND	ND

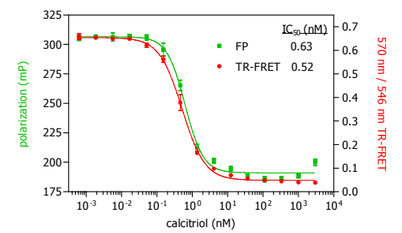
Table 2 lists the inhibition results generated using different competitive binding assays: a HAP-based ^{3}H-calcitriol displacement assay using full-length VDR, and the FP tracer displacement assay using both full-length VDR and VDR-LBD. IC_{50} values differed somewhat among formats, though the observed rank-order potency was identical. ND indicates competition not detected.

Figure 6 – TR-FRET Assay Principle



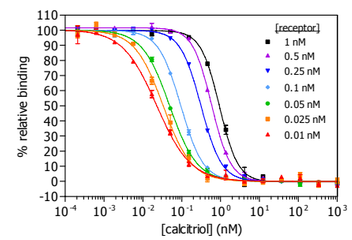
The FP-based VDR assay was converted to a time-resolved fluorescence-resonance energy transfer (FRET)-based assay through use of a glutatione transferase (GST)-tagged VDR-LBD and a terbium-labeled anti-GST antibody. When the tracer is bound to the receptor, energy transfer from the terbium to the fluorescent Fluormone VDR Red occurs, and light emission is detected at 570 nm. When the tracer is displaced by competitor, FRET is disrupted and the emission signal at 570 nm is reduced. Donor emission can be detected at either the first (495 nm) or second (546 nm) terbium emission peaks. The optical properties of time-resolved measurements can help overcome interference from background fluorescence and light-scatter caused by autofluorescent or precipitated compounds, and the ratiometric nature of FRET measurements can correct for differences in assay volumes between wells and quenching effects due to colored compounds.

Figure 7 – TR-FRET vs. FP Format



1 nM Fluormone VDR Red, 0.7 nM VDR-LBD, and 5 nM Tb-anti-GST Ab were incubated in the presence of varying amounts of calcitriol for 2 hours at room temperature. The same assay wells were read in both FP and TR-FRET modes. Similar IC_{50} results are obtained for calcitriol in either format.

Figure 8 – TR-FRET Allows for High Sensitivity Binding Studies



[VDR-LBD] (nM)	[Tracer] (nM)	Signal-to-background	Calcitriol IC_{50} (nM)	Hill Slope
1	2.2	50 ± 2	0.93	1.8
0.5	1.1	36 ± 2	0.59	1.8
0.25	0.6	20 ± 1	0.31	1.6
0.1	0.3	7.6 ± 0.5	0.095	1.5
0.05	0.2	4.1 ± 0.2	0.049	1.2
0.025	0.2	3.0 ± 0.2	0.033	1.2
0.01	0.2	1.9 ± 0.1	0.022	1.0

FP assays can be limited by the need to have sufficient amount of tracer (usually ≥1 nM for detection on most fluorescent plate readers) and a receptor concentration required to bind typically 50-80% of that total amount of tracer. By using the TR-FRET approach, we were able to use as little as 10 pM (0.4 fmol/well) receptor with 0.2 nM tracer. Lowering the tracer concentration makes the assay more sensitive to competitors (lower IC_{50}) and also enables one to lower the theoretical "tight-binding limit" imposed by the receptor concentration (i.e. it will require a competitor concentration ≥0.5 x [receptor] to occupy half of the binding sites). These effects are illustrated in the experiment above by the reduction in both calcitriol IC_{50} and hill slope values as less receptor and tracer are used. The tracer concentration selected for this experiment was based upon the apparent K_d value determined for each receptor concentration by titration of tracer (data not shown). 5 nM Tb-anti-GST antibody was used in all experiments above.

Conclusions

- We have developed a high-affinity, red fluorescent VDR ligand that can be used for the detection of novel VDR ligands using a competitive fluorescence polarization (FP) or time-resolved fluorescence resonance energy transfer (TR-FRET) assay.
- The VDR Red competition assay is non-radioactive and has a simple homogenous "mix-and-read" protocol that requires no separation of bound and free ligand.
- The VDR Red assay is robust and amenable to high-throughput screening with a 4-hour stable read window and Z' values >0.7 (Table 1).
- The VDR Red assay predicts the rank-order binding potency of VDR ligands in a manner similar to a hydroxylapatite-based ^{3}H-calcitriol displacement assay (Table 2).
- Both full-length VDR and ligand-binding domain can be used with Fluormone VDR Red. No significant differences in ligand-binding affinities were observed between the full-length and truncated receptor (Figure 2, Table 2).
- The TR-FRET format offers the optical advantages of time-resolved measurements as well as the sensitivity to reliably measure binding using sub-nanomolar amounts of receptor and tracer. This allows for resolution of the binding affinities of tight-binding competitors (Figures 7-8).

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