

Photocaged Agonist for an Analogue-Specific form of the Vitamin D Receptor

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Nuclear hormone receptors (NHRs) represent a diverse class of ligand-dependent transcriptional regulators. NHRs that have been rendered functionally inactive due to mutations that abrogate proper ligand binding can often be rescued by appropriately designed hormone analogues. The analogue-specific receptor–ligand pairs provide an ideal platform from which to develop

new chemogenomic tools for the spatial and temporal control of gene expression. Here, we describe the synthesis and in vitro assessment of a photocaged VDR agonist specific to a mutant NHR that is associated with vitamin D-resistant rickets. The results provide insight into the utility of the agonist as a potential tool for photoinduced gene patterning.

A surprising outcome of the human genome project was the conclusion that, despite the complexity of human physiology, the genome has a relatively terse cache of open reading frames by which the proteome is expected to be encoded.^[1] As many genes, transcripts, and encoded proteins impart their influences upon the development of multicellular organisms through unique spatial and temporal patterns of expression and post-translational modifications, genetic manipulation by “all-or-nothing” genetic knock-outs is increasingly being ruled out, as a single protein can impart multiple functionalities at discrete developmental milestones.^[2] These pattern-dependent functions necessitate the development of chemical tools for addressing the study of genetic and proteomic phenomena in a spatially and temporally discrete manner.^[3]

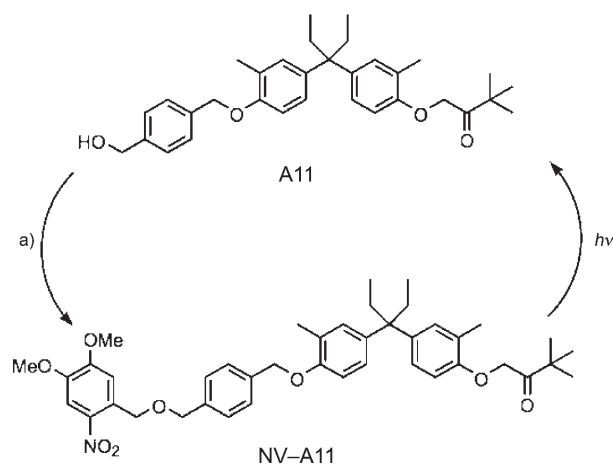
Nuclear hormone receptors (NHRs), a class of transcription factors that conditionally influence gene transcription in response to hormone–ligand binding, have been extensively studied and have been invaluable in activating and controlling eukaryotic transgene expression.^[4] We have recently pioneered an approach for regulating the spatial and temporal patterns of genes under NHR control with photocaged hormones, in which the modified ligand is unable to bind or activate its target NHR until, upon light irradiation, a photolabile protecting group is liberated to unmask a functional NHR ligand. Unlike photocaged nucleic acids or proteins, photocaged NHR ligands generally diffuse freely and are readily bioavailable.^[5] NHR-based systems are therefore only conditionally light sensitive after addition of caged ligand; however, the duration of transcription response is limited by the loss of ligand to diffusion out of the cell. We have succeeded in employing natural NHR constructs (for estrogen, retinoids, and thyroid hormone) to enforce conditional gene control with synthetic ligands.^[5] In the case of our construct under the control of the thyroid NHR, we were able to achieve a robust and sustained gene response for more than 36 hours following irradiation of a photocaged agonist.^[5b] Long-duration transcription response from intracellularly released ligands is not controlled by slow export of free ligand but rather by slow ligand off-rates and/or active receptor proteolysis.

Although the NHR systems provide exceptional control over targeted gene expression, there is an inherent risk of activating endogenous NHRs upon drug exposure. Ideally, a targeted “knock-in” NHR-based gene expression system should be under the exclusive control of a selective agonist without interference or cross talk with other hormones or hormone receptors—a concept referred to as orthogonality.^[3b,c] Additionally, the modular nature of NHRs (i.e., discrete DNA- and ligand-binding domains) makes them ideal for engineering chimeric receptors that can target alternate hormone-response element genetic tracts.^[4] This characteristic expands their utility for directed transgene activation and reduces the induction of pleiotropic effects. Heterologous constructs for this purpose, such as the insect ecdysone hormone NHR and tetracycline resistance promoter–operator systems, have been developed.^[6] However, in vivo efficacy of these systems are hampered by intrinsic systemic limitations, as ecdysone has been shown to induce multiple animal-model phenotypic changes,^[7] and tetracycline treatment is limited by toxicological concerns, including systemic antibiotic accumulation and defects in neuronal development.^[6c]

Taking a cue from nature, there exist NHR mutants that are rendered functionally inactive due to a mutation(s) within the hormone-binding pocket, which effectively abrogates proper ligand binding and/or perturbs proper conformational changes in transcriptional activation. One such example is found within the human vitamin D receptor (hVDR), whose mis-sense mutation Arg274→Leu (R274L) results in a drop in 1,25-dihydroxy vitamin D₃ (1,25D₃) potency that is >10³-fold below that of wild-type activation, and has been phenotypically associated with vitamin D-resistant rickets.^[8] Previously, our group has developed mutant-selective ligands (MSAs) that rescue, or complement, this loss-of-function mutation in the hormone-bind-

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ing pocket of VDR(R274L).^[9] Seven of these ligands exhibited potencies that were transcriptionally robust at physiologically relevant levels.^[9b] One MSA, 3-[4-(2-oxo-3,3-dimethylbutoxy)-3-methylphenyl]-3-[4-(4'-(hydroxymethyl)benzyloxy)-3-methylphenyl]pentane (ligand A11; Scheme 1), contains a *para*-hydroxymethyl group that can serve as a convenient handle for photolabile nitroveritrylation (ligand NV-A11).



Scheme 1. Scheme of NV-A11 synthesis: a) 2-nitroveratryl-bromide, $n\text{Bu}_4\text{NHSO}_4$, 10% NaOH, CH_2Cl_2 (67%), and subsequent photoactivation ($h\nu$).

To test the suitability of the A11–VDR(R274L) transgene-expression system, we monitored VDR-dependant gene expression with a luciferase reporter controlled by a vitamin D response element (VDRE) promoter. Transcriptional activity with the natural agonist, 1,25D₃, was clearly abrogated in VDR(R274L), which was rescued with A11 at physiologically relevant, low-nanomolar concentrations (50% effective concentration, EC₅₀: 15–34 nM;^[10] Figure 1 A and B). VDR(R274L) retained some activity with very high 1,25D₃ concentrations (EC₅₀ ≈ 2000 nM); however, as these concentrations exceed endogenous 1,25D₃ serum concentrations (~100 pM),^[11] the VDR(R274L) system is rendered functionally inactive in vivo in comparison to wild-type agonist. Wild-type hVDR showed some activity with A11 at slightly higher concentrations (EC₅₀ 86 nM; Figure 1 A); however, transcriptional efficacy was substantially diminished, such that A11–hVDR barely elicited a transcriptional response above baseline at the EC₅₀ of A11–VDR(R274L), as shown in Figure 1A. Taken together, these data suggest the usefulness of this 1,25D₃-inactive mutant in providing a selective transgene-expression system. However, despite this selectivity, the A11–VDR(R274L) agonist–receptor pair lacked total “functional orthogonality” due to the slight overlap of hVDR and VDR(R274L) activity at higher A11 concentrations.

Compared with A11, the *in vitro* activity of NV-A11 on the VDR(R274L) receptor was reduced by more than three orders of magnitude (Figure 1 B). Additionally, it should be noted that as VDR(R274L) was insensitive to physiological levels of 1,25D₃, special hormone-stripped serum was not needed in the cell-culture media used for transgene expression. Neither did the standard calf serum-infused media have an adverse effect on

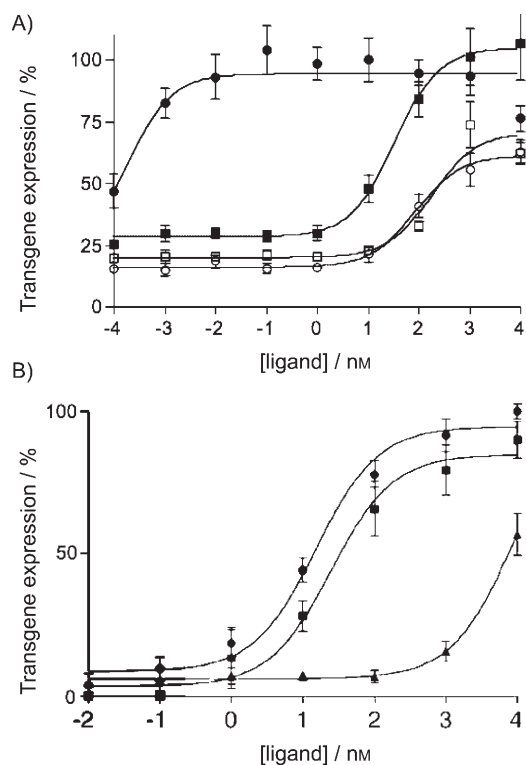


Figure 1. Profile of wild-type and mutant VDR-dependent expression of the VDRE–luciferase system in the presence of A11 and 1,25D₃ in HEK 293T cells. Ligand concentrations are represented on a logarithmic scale. A) Transcription response to hVDR with 1,25D₃ (●) and A11 (○); VDR(R274L) with 1,25D₃ (□) and A11 (■). B) Luciferase expression in response to VDR(R274L) with A11 in normal media (■), A11 in media containing charcoal-stripped serum (●), and NV-A11 in media with standard serum (▲).

A11 activation of the VDR(R274L) system (Figure 1 B). NV-A11 was photoreleased after illumination of cell culture plates with a 3 W, 365 nm long wave UV-light source. Photoactivation of NV-A11, as quantified by HPLC, was achieved efficiently with a 2 min UV exposure time, which provided >70% deprotection (elimination rate constant, $k=0.592\text{ min}^{-1}$, coefficient of determination $R^2=0.97$; Figure 2). *In vitro*, maximum VDR(R274L) transgene expression was achieved with as little as 60 s of UV exposure (three- to fivefold induction; Figure 3). Additionally, transgene expression due to UV-damage response to exposure

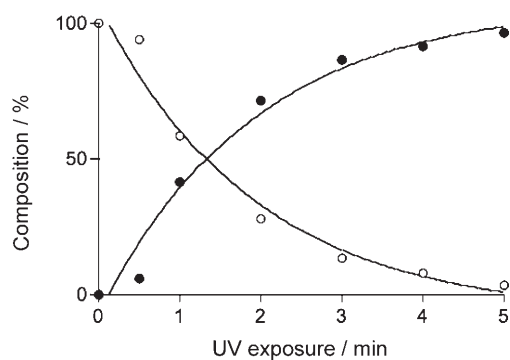


Figure 2. Quantitation of NV-A11 deprotection (○) and the emergence of A11 (●) upon UV irradiation, as monitored by HPLC.

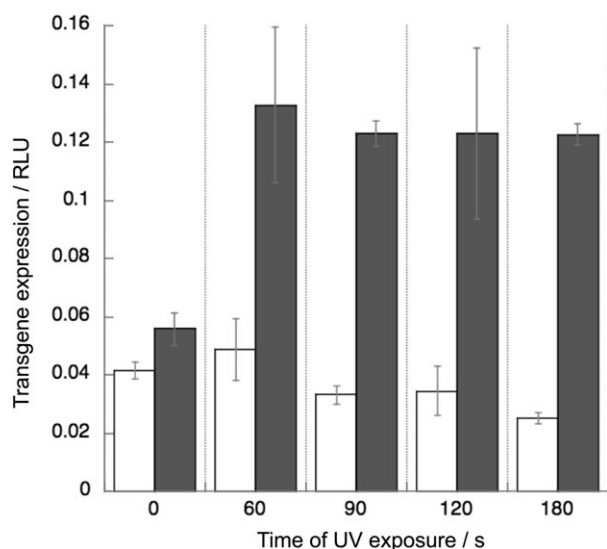


Figure 3. In vitro profile of VDRE-luciferase reporter activation with the VDR(R274L) system by using variable UV exposures times, following overnight pretreatment with (■) or without (□) 500 nM NV-A11.

was not observed; this was evident from the low background gene expression of control cells that were exposed to UV irradiation for 3 min (Figure 3).

In addition to demonstrating that NV-A11-VDR(R274L) could regulate gene expression in an exposure-dependent manner, the potential utility of this system for spatiotemporal gene expression was found to be dependent on the duration of transcription response when ligand was locally released within cells, and allowed to freely diffuse out of irradiated cells. As previously reported,^[5b] we simulate the conditions of local deprotection by preincubating HEK 293T cells with NV-A11 (100 nM), exposing the cell cultures to UV irradiation for 90 s, and promptly replacing the media with fresh media without added ligand. Whereas the combined intra- and extracellularly photoactivated NV-A11 correlated precisely with pure-agonist activation by A11, the intracellular condition alone was devoid of activity and was indistinguishable from the negative control, which was not preincubated with ligand or irradiated (Figure 4).

Previously, we noted significant differences in transient expression when using intracellularly released ligand with different NHR-based expression systems. Whereas our thyroid-hormone (TR) system achieved robust expression (>50% maximum efficacy) for more than 36 h, our retinoid system only achieved transient expression over the course of a few hours.^[5b] We attributed this discrepancy to putative on/off rates of the individual NHR-agonist complexes, and the proteolytic system that governs NHR disassembly and degradation. Additionally, while the highly robust TR system showed an almost immediate increase in transgene expression following initial ligand irradiation, the retinoid-system response was delayed by an approximately 2 h induction period. After this period, the increase in reporter-gene expression was found to be the same in both intracellular and continuous-ligand irradiation experiments. With our present VDR(R274L) system, an ap-

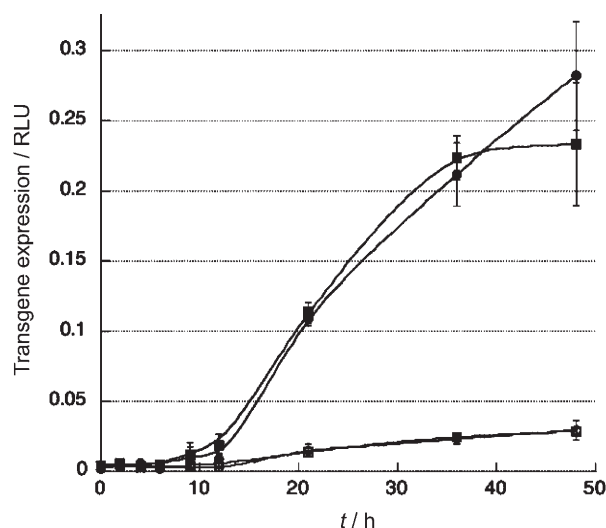


Figure 4. Time-dependence of VDR(R274L) transgene expression in HEK 293T cells. All experimental samples were UV-irradiated for 90 s. After pretreatment with NV-A11 (100 nM), overnight, the culture media was (○) or was not (●) aspirated and replaced with fresh media. As experimental controls, cells were not pretreated with NV-A11, and were (■) or were not (□) continuously exposed to A11 (100 nM).

preciable increase in transcription was not evident before 10 h of continuous A11 agonist exposure to UV light (Figure 4). This result suggests a profoundly attenuated induction period within our experimental conditions. This would suggest that in our transient photoactivation trials, the transcriptional apparatus that governs the A11-VDR(R274L) system was slowly activated. As such, it is expected that by the time transcription was initiated, the concentration of available photoactivated A11 had already dissipated and diffused freely out of the cells, so that A11 was far below the threshold concentration for activation within the cells. These results provide a general caveat that governs the development of NHR-based transgene-activation systems. It remains to be seen whether radical differences in NHR activity are solely a product of the NHR model itself or an artifact of various accessory proteins across different cell lines. A clearer understanding of these issues is expected in the near future as additional MSA-mutant NHR pairs have been developed.^[12]

The selective, yet protracted nature of our MSA-NHR system provides an intriguing corollary on its utility, despite the absence of effective light-activated gene patterning. Whereas the latency of our A11-VDR(R274L) construct was unforeseen, the selectivity and duration of transcriptional dormancy might be well suited for alternative gene-patterning designs that require delayed transcriptional efficacies. Increasingly, the study of gene function has expanded outside modes of traditional transgenic activation (i.e., aspects of controlling immediate, ligand-enforced gene expression) towards genetic circuit engineering.^[13] The modulation of discrete time periods of expression from constructs whose actions can be either immediate or delayed (working both alone or in concert with other co-expressed transactivation systems) has resulted in a growing demand for alternative transgene paradigms based on individ-

ual promoter strengths and transcriptional attributes.^[13b] These assessments of gene-expression patterns among different types of ligand–receptor pairs provide variability in the expanding repertoire of available transgenic gene-patterning systems, and the fine-tuning of synthetic biological networks.

Experimental Section

Synthesis of NV–A11: Ligand A11 (3-[4-(2-oxo-3,3-dimethylbutoxy)-3-methylphenyl]-3-[4-(4'-(hydroxymethyl)benzyloxy)-3-methylphenyl]pentane) was synthesized as previously described by Swann et al.^[9b] 2-Nitroveratryl-bromide (47.8 mg, 0.17 mmol) was added to a stirred solution of A11 (58.0 mg, 0.12 mmol) and tetrabutylammonium hydrogensulfate (54.7 mg, 0.16 mmol) in CH₂Cl₂ (1 mL), and NaOH (1 mL, 10%). The reaction mixture was then stirred vigorously for 3 h at room temperature. The reaction mixture was then diluted with water (5 mL), extracted with CH₂Cl₂ (50 mL), washed with saturated NaCl (20 mL), dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by silica-gel column chromatography to give 53.4 mg (67% yield) NV–A11. ¹H NMR (400 MHz, CDCl₃), 7.72 (s, 1 H), 7.36–7.45 (m, 5 H), 6.87–6.94 (m, 4 H), 6.76 (d, *J* = 7.4 Hz, 1 H), 6.49 (d, *J* = 7.4 Hz, 1 H), 5.03 (s, 2 H), 4.99 (s, 2 H), 4.84 (s, 2 H), 4.70 (s, 2 H), 3.97 (s, 3 H), 3.95 (s, 3 H), 2.23 (d, *J* = 5.0 Hz, 6 H), 1.96–2.05 (m, 4 H), 1.25 (s, 9 H), 0.59 (t, *J* = 5.0 Hz, 6 H); ¹³C NMR (360 MHz, CDCl₃) 210.05, 154.50, 153.96, 153.80, 147.54, 141.52, 140.90, 137.46, 137.24, 130.95, 130.75, 130.59, 127.78, 127.37, 126.10, 126.05, 125.93, 122.74, 110.22, 110.14, 109.50, 107.86, 72.95, 69.60, 69.52, 69.14, 56.43, 56.35, 48.41, 43.23, 29.29, 26.36, 16.66, 8.46; HRMS calcd for C₄₂H₅₁NO₈: 720.3512 [*M*+Na], found: 720.3753.

Cell culture, transfection, and luciferase assay: HEK 293T cells (human embryonic kidney cell line) were grown and maintained in phenol-red free DMEM with sodium pyruvate (Mediatech; Herndon, VA) supplemented with L-glutamine (2 mM), defined bovine calf serum (10%; HyClone; Logan, UT), and gentamycin (50 µg mL⁻¹) at 37 °C, 5% CO₂. The day preceding transfection, cells were seeded at 30 000 per well (24-well culture dish; 500 µL media each well).

For experiments with media devoid of vitamin D, 3 h before transfection, media was aspirated and replaced with the same formulation, but containing a 3× charcoal-stripped FBS serum (10%) substitution. VDR plasmids employed in this experiment have been previously described.^[9] Cells were transfected, in triplicate, with reporter plasmid luciferase-plus gene (138 ng) under vitamin D response element control (VDRE–Luc⁺), hVDR or VDR(R274L) hormone receptor constructs (75 ng), and *Renilla* luciferase control plasmid (27 ng) per well by using the calcium phosphate precipitation protocol. Six hours after transfection, the calcium phosphate precipitate was removed by aspiration and replaced with media (500 µL) with the appropriate concentration of ligand. After 24 h incubation all wells were aspirated and the media was renewed (500 µL) with the appropriate ligand concentration. The experiment was concluded 48 h after transfection, wherein all wells were aspirated, lysed, and assayed.

Luciferase activity was assayed with the dual-luciferase-reporter assay system (Promega; Madison, WI, USA) by using an 1450 Microbeta luminescence counter (Perkin–Elmer Life Science; Boston, MA, USA). Culture wells were treated with passive lysis buffer (100 µL), agitated for 15 min on ice, transferred to a 96-well opaque-bottomed microtiter plate (15 µL), and assayed as described. Reporter activity, in relative light units (RLUs), was defined

as the proportion of luminescence generated by the reporter luciferase normalized with respect to the *Renilla* control. The resulting data were analyzed by using Microsoft Excel (mean and standard errors) and GraphPad Prism (nonlinear regression analysis).

HPLC quantitation of NV–A11 light-irradiated deprotection: Quantitation of NV–A11 deprotection upon UV irradiation was monitored with HPLC. Under dark-room conditions, NV–A11 (100 µM) in ethanol (150 µL total volume) was irradiated by using a Spectroline XX-15A long wave 3 W, 365 nm UV lamp through a 4 mm thick glass plate and the polystyrene lid of a 24-well plate, which contained the samples, for different time periods. The irradiated samples (5 µL) were resolved on an Alltech Econosil 5U C18 column (250×4.6 mm) with a fitted guard column by using a linear gradient of acetonitrile/water (60–100%); 1.0 mL min⁻¹, A₂₈₀. Data were analyzed by using GraphPad Prism (exponential decay).

Light activated gene patterning: Transfection conditions were identical to those described above. Under dark-room conditions, transfected cells were pretreated with NV–A11 (500 nM; 500 µL total volume), overnight, irradiated by using the above-mentioned protocol at various exposure times (0, 60, 90, 120, and 180 s), and incubated for 36 h. After incubation cells were lysed and assayed as mentioned above. Control samples were irradiated without being pretreated with NV–A11.

For the simulation of local ligand release, cells were pretreated with NV–A11 (100 nM) under dark-room conditions, overnight, and were either washed (media aspirated at time of irradiation and replaced with fresh media without added ligand) or unwashed (no media change). As experimental controls, cells that were not pretreated with NV–A11 were and were not continuously exposed to pure agonist A11 (100 nM) at *t* = 0. All experimental samples were UV-irradiated through polystyrene culture-plate lids for 90 s after incubation with ligand and triplicate wells were aspirated and lysed at time points *t* = 0, 2, 4, 6, 9, 12, 22, 36, and 48 h after irradiation. Luciferase activities were determined as described above.

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