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Modulation of Transcription Mediated by the Vitamin D Receptor and the Peroxisome Proliferator-Activated Receptor δ in the Presence of GW0742 Analogs

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Abstract

Herein we describe the evaluation of GW0742 analogs in respect to their ability to modulate transcription mediated by the Vitamin D Receptor (VDR) and the peroxisome proliferator activated receptor (PPAR)δ. The GW0742 analog bearing carboxylic ester functionality in place of the carboxylic acid was partially activating both nuclear receptors at low concentration and inhibited transcription at higher compound concentrations. The GW0742 alcohol derivative was more active than the ester in respect to VDR but less active in regard to PPARδ. Importantly, the alcohol derivative was significantly more toxic than the corresponding acid and ester.

Keywords: Vitamin D; Peroxisome; GW0742 Analogs

Introduction

Nuclear Receptors (NR) are one of the most important current drug targets [1]. The development of new synthetic NR ligands for various diseases has been complicated by the fact that once affinity towards a certain receptor has been accomplish by medicinal chemistry a second level of selectivity among NRs has to be optimized in order to specifically modulate the gene regulation mediated by a particular NR. Recently, coregulators have been identified as master regulator of NR-mediated gene regulation [2]. Therefore, NR ligand interactions with NRs can influence gene regulation by modulating the specific recruitment of co-regulators [3].

In respect to 48 identified NRs and more than 300 coregulators there are more than 10,000 possible interactions between NRs and coregulators. Limited biochemical approaches to determine the influence of NR ligands in regard to these interactions have been reported [4,5]. A virtual screening approach was introduced by Schapira et al. [6] using a model based on NR ligands bound to NR ligand binding domains to predict the affinity of small molecules towards different NRs. Based on this approach it seems feasible to predict small molecule modulation of NR-coregulator interactions based on crystal structures of NRs bound to coregulators.

Generally, coactivators bind NRs in the presence of ligand whereas corepressors interact with NRs in the absence of ligand [7,8]. Corepressor binding has also been observed in the presence of NR antagonists [9]. Fortunately, crystal structures of NRs bound to antagonist or agonist in the presence of coregulator peptides are available to develop a model to predict NR-coregulator modulation of new synthetic NR ligands [10-13]. However, for the vitamin D receptor (VDR), crystal structures of VDR-corepressors complexes are still missing. Although many VDR crystal structure in the presences of antagonist have been solved [14], it seems that the antagonistic structure of VDR is induced by corepressors rather than the ligand.

Recently we have introduced GW0742, which was developed by GlaxoSmithKline as highly a selective agonist for the peroxisome proliferator activated receptor δ [15], as a novel antagonist for VDR [16]. Subsequently, we determined the activity of GW0742 for 12 nuclear receptors in the antagonist and agonist mode to determine the selectivity of GW0742 towards different nuclear receptors. Herein we describe the ability of GW0742 analogs to mediate agonistic and antagonistic effects together with the nuclear receptors VDR and PPAR δ.

Materials and Methods

Reagents

1,25-$(OH)_2D_3$ (calcitriol) was purchased from Endotherm. GW0742 was purchased from Tocris.LG190178 was synthesized using a published procedure [15].

Labeled coactivator peptides

The peptide SRC2-3 (CLQEKHRILHKLLQNGNSPA) [16], was purchased and labeled with the cysteine-reactive fluorophore, Alexa Fluor 647 maleimides, in a 50:50 DMF/PBS mixture. After purification by high performance liquid chromatography, the corresponding labeled peptide was dissolved in DMSO and stored at -20°C.

Protein expression and purification

The VDR-LBDmt DNA was kindly provided by Nandhikonda, et al. [17] and cloned into the pMAL-c2X vector (New England Biolabs). A detailed expression and purification protocol for VDR was reported previously [16].

Fluorescence polarization assay with VDR–SRC2-3

Agonistic activity and competitive inhibition were studied using a FP assay. This assay was conducted in 384-well black polystyrene plates (Corning) using a buffer [25 mM PIPES (pH 6.75) 50 mM NaCl, 0.01%

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NP-40, 2% DMSO, VDR-LBD protein (0.1 µM), LG190178 (0.75 µM), and Alexa Fluor 647-labeled SRC2-3. Small molecule transfer into a 20 µL assay solution was accomplished using a stainless steel pin tool (V&P Scientific), delivering 100 nL of the serially diluted compound solution (1:3 dilution starting at a 10 mM concentration).Fluorescence polarization was detected after initial mixing at excitation and emission wavelengths of 650 and 665 nm (Alexa Fluor 647). Three independent experiments were conducted in quadruplicate, and data were analyzed using nonlinear regression with a variable slope (GraphPadPrism).

Transcription assays

HEK 293T cells (ATTC) were cultured in 75 cm^2 flasks using DMEM/High Glucose (Hyclone, catalog no. SH3024301), nonessential amino acids, HEPES (10 mM), penicillin and streptomycin, and 10% dialyzed FBS (Invitrogen, catalog no. 26400-044). At 70-80% confluency, 2 mL of untreated DMEM containing 1.56 µg of NR plasmid, 16 µg of a luciferase reporter gene, Lipofectamine LTX (75 µL), and PLUS reagent (25 µL) was added. After 16 h, the cells were harvested with 0.05% trypsin (3 mL) (Hyclone, catalog no. SH3023601), added to 15 mL of DMEM/High Glucose (Hyclone, catalog no. SH3028401), nonessential amino acids, sodium pyruvate (1 mM), HEPES (10 mM), penicillin and streptomycin, and 2% charcoal-treated FBS (Invitrogen, catalog no. 12676-011), and spun down for 2 min at 1000 rpm. The cell were re-suspended in the same medium and plated in sterile cell culture-treated black 384-well plates with an optical bottom (Nunc, catalog no. 142761) at a concentration of 15000 cells/well, which had been previously treated with a 0.25% solution of Matrigel (BD Bioscience, catalog no. 354234). After 2 h, plated cells were treated with small molecules in vehicle DMSO, followed by 16 h incubation. The NR agonists used for VDR and PPARδ were 1, 25-$(OH)_2D_3$(10 nM) and GW7647 (30 nM), respectively. Transcription was assessed using Bright-Glo (Promega). Cell viability was assessed using CellTiter-Glo (Promega) for identically treated cells. Two independent experiments were conducted in quadruplicate, and data were analyzed using nonlinear regression with a variable slope (GraphPad Prism).

Results and Discussion

The activities of compounds 1-3 with respect to VDR were determined using a reported fluorescence polarization assays employing recombinant VDR-LBD, Alexa Fluor 647 labeled SRC2-3 peptide, and the synthetic VDR ligand LG190178 [17]. The results are presented in Table 1.

A VDR-LBD concentration used was 0.1 µM. Inhibition of VDR-SRC2-3 interaction in the presence of LG190178 (0.75 µM). Three independent experiments were conducted in quadruplicate and data were analyzed using a nonlinear regression with a variable slope (GraphPad Prism).

Compound	R	R_1	Agonist EC50 (µM)	Antagonist[a] IC50 (µM)
1 GW0742	F	CH_2COOH	Inactive	7.73 ± 1.68
2	H	CH_2COOCH_3	Inactive	>30
3	H	CH_2CH_2OH	Inactive	9.03 ± 5.5

Table 1: Summary of EC_{50} and IC_{50} values of compounds 1-3 for VDR determined by fluorescence polarization.

Compounds 1-3 possess no agonistic activity;thus they do not support the interaction between VDR-LBD and coactivator peptide SRC2-3.The antagonistic behavior of all compounds was determined in the presence of VDR agonist LG190178 [18]. Compound 1, GW0742, was most the active inhibitor with an IC_{50} of 7.73 ± 1.68 µM in the presence of 100 nM VDR-LBD. We observed that higher concentrations of VDR-LBD result in ligand depletion and therefore higher IC50 values for instance the recently reported IC_{50} of 27.2 ± 2.7 µM for GW0742 in the presence of 600 nM VDR-LBD [16]. Interestingly, compound 2 bearing carboxylic ester functionality instead of the carboxylic acid is significantly less active. The corresponding alcohol 3 however has a similar inhibitory activity as GW0742 with an IC_{50} of 9.03 ± 5.5 µM.

To further explore the biological role of compounds 1-3, transcription assays mediated by VDR and PPARδ were employed using transiently transfected HEK293-T cells [17]. The results are summarized in Table 2.

As expected, GW0742 was inactive as a VDR agonist and could inhibit VDR-mediated transcription with an IC_{50} value of 12.7 ± 8.0 µM (Table 2). Furthermore, we could confirm the activation of PPARδ at nanomolar concentrations of GW0742 (EC50 3.5 ± 0.31 nM) and inhibition of PPARδ-mediated transcription at higher concentration (IC_{50} 3.9 ± 2.41 µM) [16]. Interestingly, compounds 2 and 3, which were not able to initiate the interaction between VDR-LBD and SRC2-3 peptide (Table 1) exhibited partial VDR agonistic effects at concentrations between 110-150 nM (Table 2, entries 2 and 3). Compound 3 activated the VDR-mediated transcription with a 38% efficacy in respect to VDR agonist calcitriol and an affinity of 0.12 ± 0.03 µM (EC_{50}). For the activation of PPARδ-mediated transcription compound 2 was superior to compound 3 with an EC50 of 3.9 ± 0.38 nM and an efficacy of 35% in comparison to GW0742. Importantly, compound 2 and 3 inhibited PPARδ and VDR-mediated transcription at sub-micromolar concentrations. In addition, the toxicity of analogs 2 and 3 is more pronounced than that of GW0742. Compound 2 has three-fold selectivity towards the inhibition of PPARδ-mediated transcription with an IC_{50} of 0.26 ± 0.12 µM and compound 3 is two-fold more active to inhibit VDR-mediated transcription with an IC_{50} of 0.36 ± 0.055 µM.

Overall, we demonstrated agonistic behaviors of GW0742 and its analogs at lower concentrations for PPARδ and inhibition of PPARδ-mediated transcription at higher concentrations. In addition, GW0742 analogs 2 and 3 exhibited a similar behavior for VDR but at significantly higher concentrations for the partial agonist effect. The toxicity of both compounds is significant and may play a role in the relative low inhibition of transcription for the both receptors.

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Compound	R[a]	R_1[a]	VDR EC50 (µM)	VDR IC_{50} (µM)	PPARδ EC_{50} (nM)	PPARδ IC_{50}(µM)	Toxicity LD_{50} (µM)
1 GW0742	F	CH2COOH	Inactive	12.7 ± 8.0	3.5 ± 0.31	3.9 ± 2.4	>50
2	H	CH2COOCH3	0.15 ± 0.08 (11%)[b]	0.95 ± 0.30	3.9 ± 0.38 (35%)[c]	0.26 ± 0.12	5.47 ± 3.3 (70%)[d]
3	H	CH2CH2OH	0.12 ± 0.03 (38%)[b]	0.36 ± 0.055	40 ± 19 (13%)[c]	0.63 ± 0.22	1.73 ± 0.14

[a]for structure see Table 1; [b]percent partial VDR activation in reference to calcitriol; [c]Percent partial PPARδ activation in reference to compound GW0742; [d]Percent partial toxicity

Table 2: Summary of transcriptional activation and deactivation mediated by VDR and PPARδ in the presence of GW0742 analogs.

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