Bioassay for the Identification of Natural Product-Based Activators of Peroxisome Proliferator-Activated Receptor-γ (PPARγ): The Marine Sponge Metabolite Psammaplin A Activates PPARγ and Induces Apoptosis in Human Breast Tumor Cells

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Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor (NHR) family, are ligand-activated transcription factors. Ligands (agonists) of PPARγ have been shown to inhibit growth, promote terminal differentiation, and induce apoptosis in human breast tumor cells. A cell-based reporter assay was developed to examine extracts of terrestrial and marine organisms for the ability to activate PPARγ. Bioassay-guided fractionation and isolation of an active extract from *Pseudoceratina rhax* yielded the known histone deacetylase (HDAC) inhibitor psammaplin A (**1**). Compound **1** activates PPARγ in a MCF-7 cell-based reporter assay and induces apoptosis in human breast tumor cells *in vitro*. Molecular modeling studies suggest that **1** may interact with binding sites within the PPARγ ligand-binding pocket. Therefore, in addition to its known effects on HDAC-mediated processes, activation of PPARγ-regulated gene expression may play a role in the ability of **1** to induce apoptosis.

Peroxisome proliferator-activated receptors (PPARs) were first identified as the nuclear receptors that mediate the pleiotropic effects of peroxisome proliferators.[1] As indicated by the name, peroxisome proliferators are a collection of structurally diverse chemicals (i.e., steroids, lipids, hypolipidemic drugs, plasticizers, etc.) that cause hepatomegaly in rodents.[2] The three known PPAR isotypes PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3) are involved in numerous biological processes that range from lipid, glucose, and energy homeostasis to inflammation and wound repair.[2−4] Following activation by ligands, PPARs heterodimerize with retinoid X receptor (RXR or NR2B), bind to the peroxisome-proliferator response element (PPRE) present in the promoter regions of target genes, recruit co-activators, and activate transcription of target genes.[2−4]

The PPARγ isotype is highly expressed in adipose tissue and plays an important role in adipocyte differentiation.[5] The best known therapeutic application of PPARγ ligands is the use of thiazolidinediones (TZDs) as insulin sensitizers to treat type 2 diabetes.[6] In addition to the TZDs [i.e., rosiglitazone (Avandia) and pioglitazone(ACTOS)], PPARγ agonists also include a series of synthetic tyrosine derivatives (i.e., GW7845, GW1929, etc.), semisynthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), naturally occurring eicosanoids (i.e., 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2), polyunsaturated/hydroxylated fatty acids (i.e., 15-HETE, 13-HODE, etc.), and oxidized alkyl phospholipids (i.e., hexadecyl azelaoyl phosphatidylcholine, or azPC).[7] Since Tontonoz and co-workers discovered that PPARγ agonists induce terminal differentiation of human liposarcoma cells *in vitro*,[8] a number of studies have demonstrated that PPARγ agonists of different structural classes can inhibit growth, cause terminal differentiation, and induce apoptosis of human tumor cells derived from various tissues *in vitro* and *in vivo*.[9] However, other studies suggested that PPARγ activation may exert the opposite effect: growth promotion.[10] Experimental design and data interpretation of these seemingly controversial studies have been discussed extensively in several recent reviews.[11] Factors such as genetic predisposition, interactions between PPARγ and other signaling pathways, PPARγ-dependent and PPARγ-independent effects exerted by PPARγ agonists, concentration or dose, and the presence of endogenous PPARγ receptor and ligand(s) can all impact the final outcome (growth inhibition or growth promotion of tumor cells).

Thiazolidinediones (TZDs) such as pioglitazone (ACTOS) and rosiglitazone (Avandia) have been used clinically for years to treat type 2 diabetes. The growth inhibitory effects exerted by these PPARγ agonists in preclinical studies prompted several clinical trials to evaluate the therapeutic potential of these oral antidiabetic drugs for cancer.[12] Encouraging results were obtained from clinical trials that used troglitazone to treat liposarcoma[12a] and prostate cancer[12c,d] and trials that examined pioglitazone as part of a combination chemotherapy to treat advanced vascular tumors, melanoma, and soft tissue sarcoma.[12f,g] However, rosiglitazone was found ineffective for the treatment of liposarcoma,[12b] and troglitazone failed in advanced colon and breast cancer trials.[12e,h] Among the TZD class of oral antidiabetic drugs evaluated, troglitazone (Rezulin) was withdrawn from the market for diabetes treatment due to rare, idiosyncratic life-threatening hepatitis.[12i] The outcomes from these clinical trials suggest that PPARγ agonists may have therapeutic applications for cancer, although the TZD class of PPARγ agonists appears to have limited chemotherapeutic potential. Therefore, the discovery of novel high-affinity PPARγ activators would provide new "chemical ideas" from which to design novel classes of antitumor agents.

Results and Discussion

On the basis of results that indicate PPARγ agonists can suppress human breast tumor cell growth *in vitro* and *in vivo*[9a,b,13] and prevent chemically induced carcinogenesis of the breast in rats,[14] the drug discovery effort reported herein was directed at the identification of novel natural product-derived PPARγ activators for breast cancer treatment. Established cell lines derived from human tumors have been used extensively as experimental models of neoplastic diseases for both drug discovery and early stage drug evaluation. Two well-characterized human breast carcinoma cell lines, MCF-7 and MDA-MB-231, were selected as *in vitro* models to develop high-

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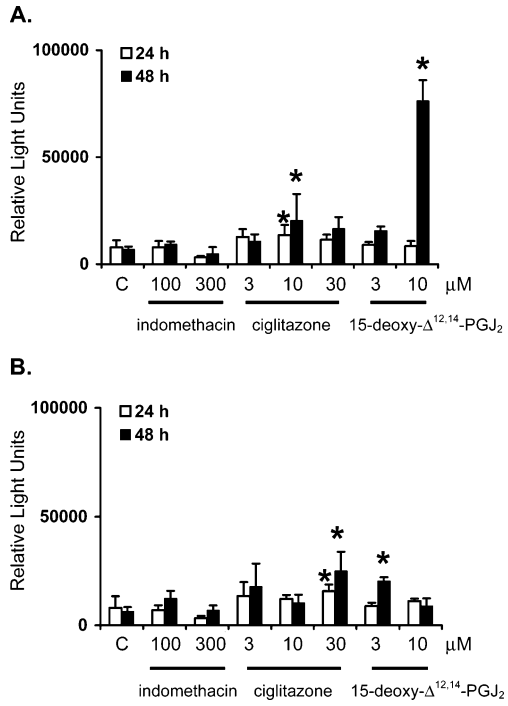


Figure 1. Effects of indomethacin, ciglitazone, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 on the activation of PPARγ in MCF-7 (A) and MDA-MB-231 (B) cell-based reporter assays. Cells that were transiently transfected with the pPPRE-aP2-tk-luc reporter and the PPARγ expression construct pCMV-rPPARγ were exposed to test compounds at the indicated concentrations for 24 (open bar) and 48 h (solid bar). Luciferase activities were determined following incubation. Data presented are averages from one representative experiment performed in triplicate, and the bars represent standard deviation. An asterisk (*) indicates a significance of $p < 0.05$ when compared to the untreated control "C".

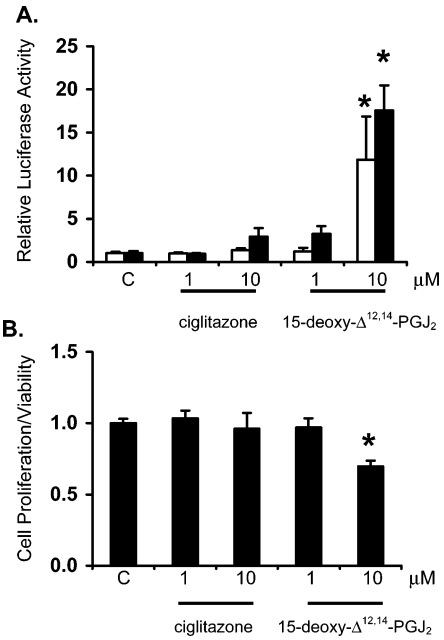


Figure 2. Ciglitazone and 15-deoxy-$\Delta^{12,14}$-PGJ_2-dependent activation of endogenous PPARγ (A) and inhibitory effects on cell proliferation/viability in MCF-7 cells (B). MCF-7 cells were transiently transfected with either the pPPRE-aP2-tk-luc reporter alone (open bar) or a mixture of pPPRE-aP2-tk-luc reporter and pCMV-rPPARγ (solid bar) (A). Luciferase activities following compound treatment for 48 h are presented as averages from a representative experiment performed in triplicate, and the bars represent standard deviation. MCF-7 cell proliferation/viability following 48 h treatment is presented in B (average + standard deviation, $n = 3$). An asterisk (*) indicates a significance of $p < 0.05$ when compared to the untreated control "C".

throughput screening (HTS) assays for functional activators of PPARγ. The MCF-7 cells depend on estrogen for growth and have been used as an *in vitro* model for early stage hormone-dependent breast tumors. The MDA-MB-231 cells do not require estrogen for growth and are highly tumorigenic in animal models. The MDA-MB-231 cells were used as an *in vitro* model for hormone-independent, advanced stage breast tumors. A luciferase reporter gene under the control of mouse fatty acid binding protein-2 (aP2, a PPARγ target gene) promoter (pPPRE-aP2-tk-luc) was employed to monitor PPARγ activity.[15] The region of aP2 promoter used in this reporter construct contains DNA sequences that specifically respond to activated PPARγ.[16] Activation of PPARγ is reflected by an increase in the luciferase activity. To optimize and standardize the screening assays, three PPARγ agonists (indomethacin, ciglitazone, and 15-deoxy-$\Delta^{12,14}$-PGJ_2) were tested in these 96-well plate-based assays. Exponentially grown MCF-7 and MDA-MB-231 cells were transfected with the pPPRE-aP2-tk-luc reporter and a PPARγ expression construct (pCMV-rPPARγ),[15] exposed to test compounds for 24 and 48 h, and the luciferase activities determined (Figure 1A: MCF-7 cells, and Figure 1B: MDA-MB-231 cells). The highest level of induction (11-fold) was observed in MCF-7 cells following a 48 h exposure to 15-deoxy-$\Delta^{12,14}$-PGJ_2 at the concentration of 10 μM. No activation was observed in the presence of indomethacin, which was reported to activate PPARγ with an EC_{50} of 50 μM in CV-1 cells.[17] The genetic differences between the African green monkey kidney derived CV-1 cells and the human breast tumor MCF-7 and MDA-MB-231 cells may cause the lack of activity for indomethacin to activate PPARγ in either breast tumor cell lines. The thiazolidinedione ciglitazone that significantly activated PPARγ at the concentration of 10 μM in CV-1 cells[7a] exhibited only a modest activity in both MCF-7 and MDA-MB-231 breast tumor cells (Figure 1). These results suggest that both MCF-7 and MDA-MB-231 cells respond similarly to PPARγ activators and MCF-7 cells generate a more robust response.

MCF-7 cells express endogenous PPARγ protein at levels significantly higher than MDA-MB-231 cells.[9b] We then tested if the endogenous PPARγ in MCF-7 cells is sufficient to activate the pPPRE-aP2-tk-luc reporter. MCF-7 cells were transfected with either the pPPRE-aP2-tk-luc reporter alone or a mixture of pPPRE-aP2-tk-luc and the PPARγ expression construct pCMV-rPPARγ. Following a 48 h incubation with the PPARγ agonists ciglitazone and 15-deoxy-$\Delta^{12,14}$-PGJ_2, the luciferase activities were determined. The pPPRE-aP2-tk-luc reporter was activated by 15-deoxy-$\Delta^{12,14}$-PGJ_2 (10 μM) even in the absence of overexpressed exogenous PPARγ (Figure 2A), suggesting that the endogenous PPARγ in MCF-7 cells is functional. Activation of PPARγ by 15-deoxy-$\Delta^{12,14}$-PGJ_2 (10 μM) is associated with a decrease in MCF-7 cell proliferation/viability (Figure 2B). A concentration−response study was performed to further characterize the effects of 15-deoxy-$\Delta^{12,14}$-PGJ_2. At the lower concentrations (3 to 30 μM), PPARγ activation correlates with the reduction in cell proliferation/viability in a concentration-dependent fashion (Figure 3A). The greatest activation was observed at the concentration of 30 μM, and this activation coincides with a significant drop in cell viability. At the highest concentration tested (60 μM), no luciferase activity was detected, and this loss of PPARγ activation was likely due to cytotoxicity. The morphological changes incurred by the treatment of MCF-7 cells with 15-deoxy-$\Delta^{12,14}$-PGJ_2 (48 h) are shown in Figure 3B. At the concentration of 30 μM, 15-deoxy-$\Delta^{12,14}$-PGJ_2 caused the adherent MCF-7 cells to become detached and round, indicative of cell death. To maintain the consistency between assays and to reduce the variation in luciferase activity caused by cell death, a

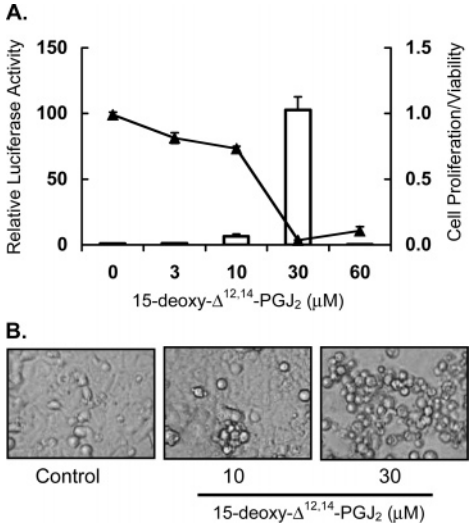
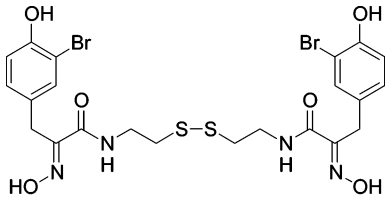


Figure 3. Concentration−response of 15-deoxy-$\Delta^{12,14}$-PGJ_2 on PPARγ activation and MCF-7 cell proliferation/viability (A) and morphological changes (B). MCF-7 cells transiently transfected with the pPPRE-aP2-tk-luc reporter were exposed to 15-deoxy-$\Delta^{12,14}$-PGJ_2 at the concentrations indicated for 48 h. Luciferase activities from one representative experiment performed in quadruplicate are presented as averages (open bar), and the bars represent standard error (A). Cell proliferation/viability following 48 h treatment is presented as a line with solid triangles (A). Representative bright field images were obtained at 100× magnification (B).

suitable positive control should afford significant activation of PPARγ while exerting minimal cytotoxicity. Therefore, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (10 μM) was selected as the positive control for the screening assays.

To discover novel PPARγ activators, the MCF-7 cell-based pPPRE-aP2-tk-luc reporter assay was used to examine extracts from plants and marine organisms for their ability to activate PPARγ. The crude extracts were tested at the final concentration of 30 μg mL^{-1}. Extracts that activated the reporter to a level ≥50% of that produced by the positive control (10 μM 15-deoxy-$\Delta^{12,14}$-PGJ_2) were considered positive. Among the 2688 extracts from marine organisms that were examined, the only active extract was from the sponge *Pseudoceratina rhax* (Aplysinelliae) that was obtained from the National Cancer Institute Open Repository. Bioassay-guided chromatographic fractionation of the *P. rhax* lipid extract (4 g) yielded a known compound, psammaplin A (**1**). The IR, UV, and ^1H and ^{13}C NMR spectroscopic data of **1** are in agreement with the previously reported values for *E*,*E*-psammaplin A.[18] The *E*,*E*-geometry of the oxime moieties was determined on the basis of the unique ^{13}C chemical shifts for C-7 and C-7′ that coincide at δ 28.8.[18b]



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A concentration−response study was performed to characterize the effect of **1** on PPARγ activation and cell proliferation/viability in MCF-7 cells. In the pPPRE-aP2-tk-luc reporter assay, maximum activation was achieved at the concentration of 10 μM, and the EC_{50} was shown to be 5.7 μM (Figure 4A). The activation of PPARγ correlates with a significant reduction in cell proliferation/viability with an IC_{50} of 5 μM (Figure 4A).

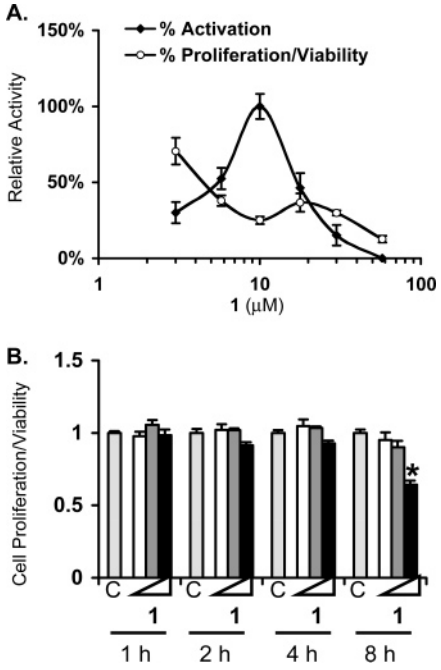

Figure 4. Concentration−response of **1** on PPARγ activation and cell proliferation/viability (A) and effects of short exposure to **1** on cell proliferation/viability in MCF-7 cells (B). The PPARγ activation and cell proliferation/viability studies were performed similarly to that described in the Figure 3 legend. Data shown are averages from one representative experiment performed in triplicate, and the bars represent standard deviation (A). For short exposure study, MCF-7 cells were exposed to **1** at a concentrations of 3 μM (open bar), 10 μM (gray bar), and 30 μM (black bar) for 1, 2, 4, and 8 h. After removal of the compounds, the cells continued to grow for 96 h in regular culture media and cell viability was determined by the neutral red method. Data shown are averages from one representative experiment performed in triplicate, and the bars represent standard deviation (B). An asterisk (*) indicates a significance of $p < 0.05$ when compared to the untreated control "C" for each time point.

Psammaplin A was initially isolated from an unidentified sponge,[18b] *Thorectopsamma xana*,[19a] and *Psammaplysilla* sp.[19b] The reported IC_{50} values for **1** to suppress tumor cell growth range from 0.39 to >30 μM, depending on the specific tumor cell line.[19b,20] To examine the effects of **1** on cell proliferation/viability following a short exposure, MCF-7 cells were exposed to **1** at the concentrations of 3, 10, and 30 μM for 1, 2, 4, and 8 h. The conditioned media were replaced by test compound-free culture media at the end of incubation, and the cells were allowed to grow for another 4 days. At the highest concentration tested (30 μM), **1** reduced cell proliferation/viability by 36% following an 8 h treatment (Figure 4B). At the concentration of 10 μM, no effect on cell proliferation/viability was observed following any of the short exposures (Figure 4B), although this same 10 μM concentration was previously shown to exhibit the greatest PPARγ activation and a 62% reduction in cell proliferation/viability following a 48 h treatment (Figure 4A). These results suggest that the PPARγ activation associated cytostatic/cytotoxic activity of **1** requires extended exposure. Most of the published studies have only used cell viability assays to examine the effects of **1** on tumor cell proliferation/viability.[19b,20] Therefore, a morphological study was performed to examine the effects of **1** on MCF-7 cells. Following 4, 8, and 16 h compound treatment, MCF-7 cells were fixed and the nuclei stained with DAPI (4′,6-diamidino-2-phenylindole), a fluorescent dye that binds DNA. Taxol (paclitaxel, 30 nM) was used as a positive control. One of the events that occurs during the process of apoptotic cell death is chromosome fragmentation, which can be observed microscopically by the appearance of apoptotic body formation. Taxol treatment caused

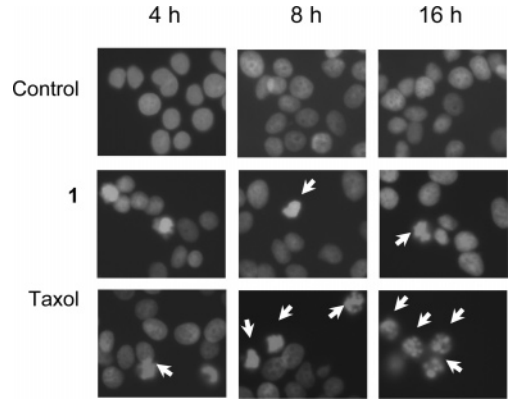

Figure 5. DAPI staining of MCF-7 cells following treatment with **1**. The cells were exposed to **1** (10 μM) or taxol (30 nM) for 4, 8, and 16 h. Apoptotic cells with fragmented nuclei are highlighted with an arrow.

apoptosis in MCF-7 cells after 4 h, and the percentage of apoptotic cells increased as the incubation time increased (Figure 5). Compound **1** induced apoptosis in MCF-7 cells after 8 h treatment, and an increase in apoptotic cells was observed at 16 h (Figure 5). Similar results were observed in human breast carcinoma T47D cells following exposure to **1** (data not shown).

Proposed binding interactions of **1** and 15-deoxy-$\Delta^{12,14}$-PGJ_2 with the PPARγ ligand binding domain based on the docking studies are shown in Figure 6. Both compounds appear to have significant hydrophobic interactions with Leu255, Leu270, Ile281, Gly284, Val339, Met340, and Ile341. The carboxylic group of 15-deoxy-$\Delta^{12,14}$-PGJ_2 seems to be involved in hydrogen-bonding interactions with His449 and Tyr473 near the AF-2 helix. Similarly, hydrogen bonds between **1** and several binding site residues such as Glu259, Gln286, and Ser342 are apparent in the proposed binding mode. In previous structure−activity relationship (SAR) and modeling studies involving a series of 15-deoxy-$\Delta^{12,14}$-PGJ_2-based ligands,[21] as well as several dimeric ligands,[22] similar hydrophobic and hydrogen-bonding interactions have been suggested to be critical for PPARγ binding.

Among the reported bioactivities attributed to psammaplin A (**1**), the most important one is the ability of **1** to inhibit the enzymes that deacetylate histone proteins, known as histone deacetylases (HDAC) (IC_{50} 4.2 nM, *in vitro* cell-free enzyme assay).[23] Several HDAC inhibitors are currently undergoing clinical trials as potential molecular-targeted chemotherapeutical agents for cancer.[24] Although **1** inhibited tumor growth both *in vitro* and *in vivo*, its poor physiological stability prevented its development as a drug.[23,24] The instability of **1** under physiological conditions (or poor cell membrane penetration) may explain why inhibition of HDAC in a cell-based assay requires a concentration of **1** that is 1800 times greater than the concentration required to inhibit HDAC in an enzyme-based assay.[23] For example, the AC_{50} for **1** to activate p21 promoter in a cell-based reporter assay is 7.5 μM (human colon carcinoma H1299),[23] a concentration comparable to that required to activate the pPPRE-aP2-tk-luc reporter in MCF-7 cells (EC_{50} 5.7 μM). Since molecular modeling studies suggest that **1** may interact with a significant number of binding sites within the PPARγ ligand-binding pocket, the ability of **1** to induce tumor cell differentiation and programmed cell death may be mediated, at least in part, through the activation of PPARγ-regulated gene expression. It remains possible that the ability of **1** to inhibit histone deacetylases may contribute to either the activation of PPARγ and/or the observed apoptotic process.[25] In summary, our results suggest that one of the mechanisms for psammaplin A to suppress tumor growth is through the induction of differentiation and subsequently apoptosis in breast tumor cells.

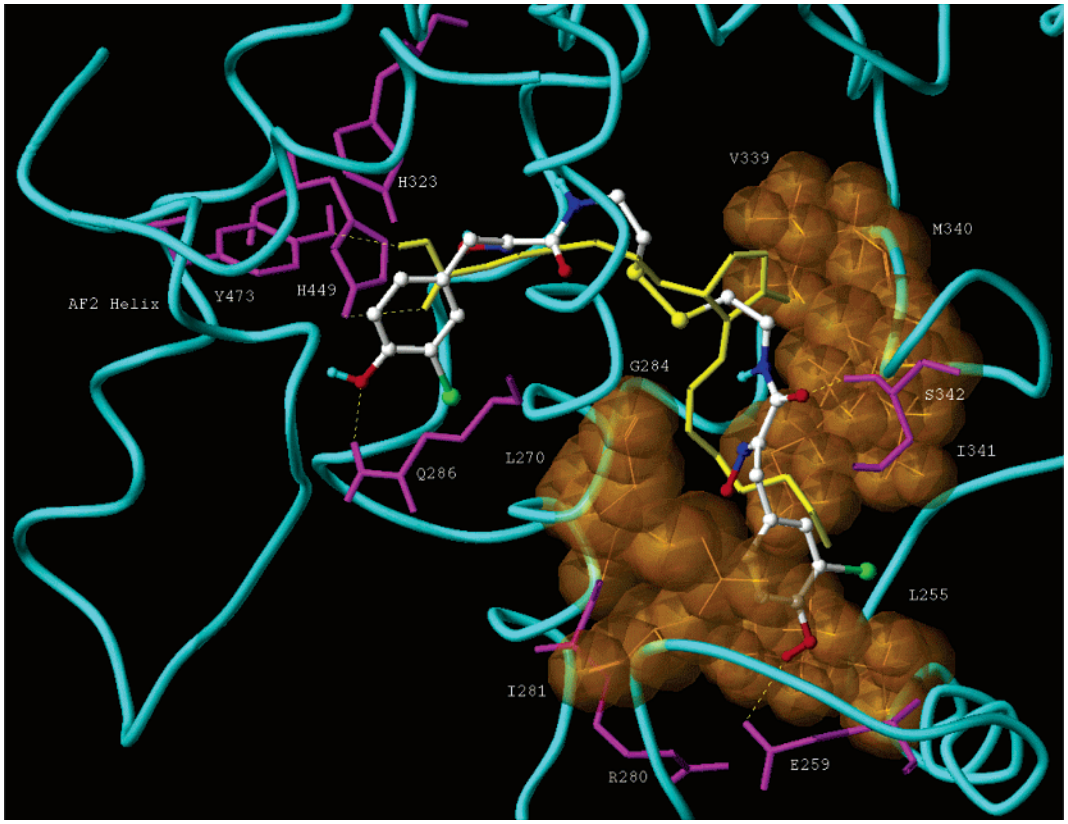

Figure 6. Proposed binding modes of 15-deoxy-$\Delta^{12,14}$-PGJ_2 and **1** in the PPARγ ligand binding domain (LBD). The known PPARγ ligand 15-deoxy-$\Delta^{12,14}$-PGJ_2 is shown as yellow sticks, and **1** as a ball-and-stick model colored by atom types. The protein is shown as a cyan ribbon, and the hydrophobic residues are depicted as transparent CPK models in orange. Other critical residues are shown as magenta sticks. Hydrogen bonds are represented as yellow dotted lines.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Rudolph Research Autopol IV automatic polarimeter. The IR spectrum was obtained using an AATI Mattson genesis Series FTIR. The ^{1}H NMR, ^{1}H−^{1}H COSY, NOESY, and HMBC spectra were recorded on a Bruker AV 500 spectrometer. The ^{13}C NMR and HMQC spectra were recorded on a Bruker DRX 500 spectrometer. Both NMR spectrometers were operated at 500 MHz for ^{1}H and 100 MHz for ^{13}C, respectively. The NMR spectra were recorded running gradients, and residual solvent peaks (d_4-MeOH, δ 4.87 for ^{1}H and δ 49.15 for ^{13}C) were used as internal reference. The ESIMS and HRESIMS data were acquired on a Bruker BioAPEX 30es mass spectrometer. TLC was performed using Merck $Si_{60}F_{254}$ or $Si_{60}RP_{18}F_{254}$ plates and visualized by spraying with H_2SO_4 in EtOH (1:1) and heating. HPLC was carried out on a Waters Millennium system with a 996 photodiode array detector. The solvents for the chemical studies were from Fisher. Indomethacin, ciglitazone, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 were from Cayman Chemical and prepared as stock solutions in DMSO. Paclitaxel (Taxol) and DAPI were from Sigma.

Sponge Material. The sponge material was obtained from the National Cancer Institute's Open Repository Program. *Pseudoceratina rhax* (Aplysinelliae) was collected in Chuuk, Federated States of Micronesia, by Dr. Patrick L. Colin on March 28, 1992. The sample was identified, frozen at −20 °C, and ground in a meat grinder. A voucher specimen was placed on file with the Smithsonian Institution in Washington, D.C. (voucher #C009533).

Extraction and Isolation. Ground sponge material was extracted with water. The residual sample was then lyophilized and extracted with CH_2Cl_2/MeOH (1:1), residual solvents were removed under vacuum, and the crude extract was stored −20 °C in the NCI repository at the Frederick Cancer Research and Development Center (Frederick, MD). A portion of the crude extract (4 g) was fractionated by VLC (Si gel) using gradient elution with hexanes, EtOAc, and MeOH. The active fraction (570 mg) eluted with CH_2Cl_2/EtOAc (1:1) and was further purified on a reversed-phase C_{18} Sep-Pack column using MeOH and H_2O. The active fraction (300 mg) that eluted with MeOH/H_2O (7:3) was subjected to further separation by reversed-phase preparative HPLC (Prodigy 5 μm, ODS, 100 Å, 250 × 21.2 mm) and isocratic solvent system CH_3CN/CH_3OH/H_2O (3:3:4) to yield **1** (60 mg, 1.5% yield).

***(E,E)*-Psammaplin A (1):** white amorphous solid; HRESIMS *m*/*z* 662.9581 (calcd for $C_{22}H_{24}Br_2N_4O_2S_2$ $[M + H]^+$ 662.9600). The spectroscopic data for **1** were in good agreement with those previously reported for *E*,*E*-psammaplin A.[18]

Cell-Based Reporter Assays. Human breast carcinoma MCF-7 and MDA-MB-231 cells (ATCC) were grown in DMEM/F12 medium (JRH Biosciences) supplemented with 10% (v/v) fetal calf serum (FCS, Hyclone), 50 units mL^{-1} penicillin G (sodium salt), and 50 μg mL^{-1} streptomycin sulfate (referred to as "Pen/Strep") (Life Technologies) in a humidified atmosphere (5% CO_2/95% air) at 37 °C. Exponentially grown cells (1×10^7) were transiently transfected by electroporation with the pPPRE-aP2-tk-luc reporter[15] and the PPARγ expression construct pCMV-rPPARγ[15] at concentrations of 50 and 10 μg mL^{-1}, respectively. Electroporation was performed in a 4 mm gap cuvette using an ECM830 square wave electroporation system (BTX Inc) at 140 V, 70 ms, 1 pulse for MCF-7 cells and 140 V, 40 ms, 2 pulses for MDA-MB-231 cells. The transfected cells were plated at a density of 20 000 cells per well into 96-well plates in a volume of 100 μL of DMEM/F12 supplemented with 10% FCS and Pen/Strep. Following a 20 h incubation, test compounds were added in a volume of 100 μL of serum-free DMEM/F12 with Pen/Strep, and the incubation continued at 37 °C. Following incubation, the conditioned media were removed, the cells were lysed, and the luciferase activity was determined using a commercial kit (Promega).

Cell Proliferation/Viability Assay. Exponentially grown cells were plated into 96-well tissue culture plates (Corning) similar to that described in the reporter assay section. Addition of test compounds was the same as described earlier. Cell proliferation/viability was determined using the neutral red method[26] with modifications as previously described.[27] The absorbance at 540 nm was measured on a microplate reader (BIO-TEK Instruments) with correction wavelength at 630 nm. For short exposure experiments, the cells were washed twice with serum-free DMEM/F12 after compound treatment. Following addition of 200 μL per well of DMEM/F12 medium supplemented with 10% FCS and Pen/Strep, the incubation continued for an additional 96 h. Cell proliferation/viability was determined using the neutral red method.

DAPI Staining. MCF-7 cells were plated at a density of 80 000 cells per well in a volume of 400 μL of DMEM/F12 medium supplemented with 10% FCS and Pen/Strep into 24-well tissue culture plates (Corning) with one coverslip inside each well. After a 16 h incubation, an equal volume of serum-free DMEM/F12 medium with Pen/Strep that contained no compound, psammaplin A (10 μM final), or taxol (30 nM final) was added. Following compound treatment for 4, 8, and 16 h, the conditioned media were removed, and the cells were fixed with MeOH for 5 min and washed twice with 1× PBS for 5 min for each wash. The coverslips were detached, one drop of Vectashield mounting medium with DAPI (Vector Laboratories) was added, and the coverslip was mounted onto microscope slides. The slides were observed using a Zeiss Axioscope fluorescent microscope with a DAPI filter at 400× magnification. Images were obtained with an AxioCam and processed with the OpenLab 3.1 software (Improvision).

Statistical Analysis. Data were compared using ANOVA and post hoc analyses using Fisher's PLSD (StatView Software Version 5.01, SAS Institute Inc). Differences were considered significant when $p < 0.05$.

Computational Methods. Computational studies were performed on a Silicon Graphics Octane-2 workstation, equipped with two parallel R12000 processors, V6 graphics board, and 512 MB memory. The crystal structure of rosiglitazone-bound PPARγ ligand binding domain (LBD) was used for the docking studies (PDB code 2PRG).[28] Hydrogen atoms were added to the protein structure, and it was subjected to preliminary minimization followed by molecular dynamics to relieve internal strain while heavy atoms were tethered to their original positions. Flexible ligand docking was performed using GOLD (version 2.2).[29] A total of 20 independent genetic algorithm (GA) runs were performed with the default standard set parameters for optimum accuracy, and the binding poses were scored using the GOLD score. Also, the docking was terminated when the top three solutions were within 1.5 Å rmsd of each other.

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