Genomic Activation of PPARG Reveals a Candidate Therapeutic Axis in Bladder Cancer

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Abstract

The PPARγ gene encoding the nuclear receptor PPARγ is activated in bladder cancer, either directly by gene amplification or mutation, or indirectly by mutation of the RXRA gene, which encodes the heterodimeric partner of PPARγ. Here, we show that activating alterations of PPARG or RXRA lead to a specific gene expression signature in bladder cancers. Reducing PPARγ activity, whether by pharmacologic inhibition or genetic ablation, inhibited proliferation of PPARG-activated bladder cancer cells. Our results offer a preclinical proof of concept for PPARG as a candidate therapeutic target in bladder cancer. Cancer Res; 77(24): 6987–98. ©2017 AACR.

Introduction

Bladder cancer is the fifth most commonly diagnosed cancer in the United States with an annual incidence of 80,000 cases and an annual mortality of 15,000. Most patients are diagnosed with noninvasive/superficial urothelial carcinoma of the bladder and respond well to transurethral resection, Bacillus Calmette-Guérin (BCG) immunotherapy, and regular cystoscopic surveillance, with a 5-year survival rate of 96%. Patients diagnosed with muscle-invasive disease have a poorer prognosis, however, with a 5-year survival rate of 35% to 70%, depending upon the extent of invasion, regional and systemic metastases, and response to therapy.

Recently, several immune checkpoint blockade drugs have been FDA-approved for treatment of urothelial carcinoma, including atezolizumab, an anti-PD-L1 antibody, in 2016 (1); nivolumab and pembrolizumab, anti-PD-1 antibodies, in 2017 (2); and avelumab and durvalumab, anti-PD-L1 antibodies, in 2017 (3, 4). These approvals mark the first new drugs for metastatic disease in nearly two decades. However, the objective response rates (defined using RECIST v1.1) were relatively low in these clinical trials, with 15%–20% overall response rate and 26%–28% response rate in PD-L1–positive patients (1, 2). Beyond checkpoint inhibitors, a number of therapies targeting specific genetic alterations, including FGFR3 alterations, mTOR pathway alterations, and DNA repair deficiencies associated with ERCC2 alterations (5–8), are under clinical evaluation.

We explored large-scale cancer genome datasets derived from patients with muscle-invasive bladder cancer with the purpose of identifying novel therapeutic targets. Hotspot mutations (p.S427F/Y) in the retinoid X receptor alpha (RXRA) gene are present in approximately 5% of bladder cancer samples (9, 10). RXRA is a well-characterized ligand-activated nuclear receptor that serves as a requisite heterodimer partner for approximately 30 nuclear receptors, including PPARA, PPARC, PPARD, RARA, RARB, VDR, TR, LXR, and PXR (11), suggesting that recurrent mutations in RXRA could impact the formation and/or function of these heterodimers and change the expression of their downstream target genes. Previous reports have shown that cancer samples containing RXRA p.S427F/Y mutations are associated with enhanced expression of genes involved in adipogenesis and lipid metabolism, including ACOX1, ACSL1, ACSL5, FABP4, and HMGS2 (9). These genes are targets of PPARγ, a member of the PPAR subfamily of nuclear receptors.

Interestingly, the PPARγ gene is locally amplified in 15% of bladder cancer samples (Supplementary Fig. S1A; ref. 12). This amplification is strongly correlated with expression of PPARγ (Supplementary Fig. S1B) as well as expression of PPARγ target genes and luminal differentiation markers such as GATA3, UPIK2, ACOX1, and UPK1A (Supplementary Fig. S1C; refs. 13–16). PPARγ is a master regulator of adipocyte differentiation and controls expression of a large set of genes involved in lipid and glucose homeostasis (12). In contrast to PPARγ’s well-characterized activity in adipocytes, however, little is known about its function in the urinary bladder and in the pathogenesis of bladder cancer.

The potential role of bladder cancer upon activation of the PPAR subfamily of nuclear receptors is controversial, and was first suggested following rodent toxicity studies testing anti-diabetic PPAR agonists in which numerous glitazar-class PPARA/PPARG...
dual agonist compounds were associated with an increased incidence of bladder cancer (17). In terms of more selective PPARG agonists, also antidiabetic drugs with insulin sensitizing activity, the carcinogenic effect in rodents was also observed with pioglitazone, which has weak PPARA activity (18), but not with rosiglitazone, which is highly selective for PPARG (17, 19). In a more sensitive, chemically-induced model using the carcinogen, 4-hydroxybutyl (butyl) nitrosamine (OH-BBN), rosiglitazone was found to potentiate urinary bladder carcinogenesis in rats compared with OH-BBN alone (20). It was originally hypothesized that the effects of PPARA/PPARG dual agonists on promoting bladder cancer was rodent-specific due to indirectly causing calcium crystal formation in the bladder, resulting in urolithiasis (21). Numerous studies have since examined the incidence of bladder cancer in humans following the clinical use of these compounds, with some detecting an increased risk and others concluding there is no increased risk (22, 23). The most comprehensive retrospective study to date showed an increase in the hazard ratio for bladder cancer with long-term, high-dose treatment with pioglitazone (24).

On the basis of the combined genetic and pharmacologic evidence, we hypothesized that activation of PPARG is oncogenic in the transitional epithelial cells of the bladder. We evaluated this by investigating the biological impact of ectopic expression of mutant alleles of RXRA and PPARG, pharmacologic ablation of RXRA/PPARG signaling using small-molecule perturbagens, and genetic ablation of RXRA and PPARG using CRISPR/Cas9 gene knockouts. Our results demonstrate an oncogenic role for PPARG in the development of luminal bladder cancer, revealing a novel axis that could be exploited for the development of targeted therapies for this disease.

Materials and Methods

Chemicals

Rosiglitazone, pioglitazone, tesaglitazar, GW9662, T0070907, LV3003, and SR1664 were purchased from Tocris Bioscience. SR2595 and SR10221 were synthesized according to published methods (25). GW1929, BADGE (Bispheonol A diglycidyl ether), SR202, and GW6471 were purchased from Sigma-Aldrich.

Cell lines

The UM-UC-9 cell line was purchased from Sigma-Aldrich. All other cell lines were obtained from the Cancer Cell Line Encyclopedia (Broad Institute, Cambridge, MA), which obtained them from the original source and performed cell line authentication (26). To reduce bias from cell culture medium, all cell lines were maintained in MEM-α medium supplemented with 10% Tet system approved FBS (Clontech).

Ectopic CDNA expression

Wild-type open reading frame (ORF) clones for RXRA and PPARGv1 were obtained from the Genomics Perturbation Platform (Broad Institute) in pDONR Gateway cloning vectors. Various mutant alleles were generated using QuickChange Site-Directed Mutagenesis (Agilent). ORFs were then subcloned into lentiviral vectors (Broad Institute) in pDONR Gateway cloning vectors. Var-ORFs were then subcloned into lentiviral vectors (Broad Institute) in pDONR Gateway cloning vectors. Various mutant alleles were generated using QuickChange Site-Directed Mutagenesis (Agilent). ORFs were then subcloned into lentiviral vectors (Broad Institute) in pDONR Gateway cloning vectors. Var-

RNA sequencing analysis

RNA was isolated using RNeasy (Qiagen) and an RNA sequencing library was prepared using NEBNext Ultra Directional RNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (New England BioLabs). Sample analysis was performed using a MiSeq System and Reagent Kits (Illumina) according to the manufacturer's protocol. Sequence data was analyzed using Firehose (Broad Institute) to map transcripts and calculate reads per kilobase million (SW780 CDNA expression) or transcripts per million (UM-UC-9). For UM-UC-9 RNA sequencing experiment, the resulting reads were used to calculate transcript abundance in units of transcripts per million (27), which were then adjusted using TM normalization (28) for comparison. Log fold-change and Mann–Whitney test significance was used to identify differentially expressed genes between the agonist and inverse-agonist–treated samples.

Western blot analysis

Cells were grown in 6-well plates and harvested using Complete Lysis-M with protease and phosphatase inhibitors (Roche Applied Science). Western blot analyses were performed using standard protocols with semi-dry transfer Trans-Blot SD (Bio-Rad), LI-COR Odyssey Blocking buffer, and imaging with LI-COR Odyssey Imaging System (LI-COR). The anti-PPARG C26H112, anti-PPARG 81B8, anti-FABP4 D25B3, and anti-CEACAM5 CB30 antibodies were obtained from Cell Signaling Technology. The anti-ACSL5 ab57210 and anti-HMCGS2 EPR8642 antibodies were obtained from Abcam. The anti-VCL (vinculin) V9264 antibody was obtained from Sigma-Aldrich. All primary antibodies were tested at 1:1,000 dilutions, with the exception of anti-VCL, which was tested at a 1:5,000 dilution. Secondary goat anti-mouse 926-68020, and goat-anti-rabbit 926-32211 antibodies were obtained from LI-COR Biosciences and used at 1:15,000 dilutions.

RT112-FABP4-NLucP reporter gene assay

Reporter cell line was generated by engineering the NanoLuc gene into the 3’ UTR of FABP4 in RT112/84 cells using CRISPR/ Cas9–guided genome engineering. Single-cell clones were isolated and the clone with the widest dynamic range selected for use. Assays were performed by seeding 384-well plates with 10,000 cells per well in MEM-α containing 10% FBS and dosing compounds at indicated concentration using the HP D300 digital dispenser (HP/Tecan). Eighteen to 24 hours after dosing with the compound, cells were assayed using NanoGlo Luciferase Assay Reagent (Promega) and plates were read using an EnVision Multi-Label Reader (PerkinElmer).

Biochemical assays

The LanthaScreen TR-FRET PPAR gamma Competitive Binding Assay and LanthaScreen TR-FRET PPAR gamma Coactivator Assay were obtained from Thermo Fisher Scientific. Assays were performed according to the manufacturer's protocol. To assay inverse-agonism, the TR-FRET PPAR gamma Coactivator assay was modified by the use of fluorescently labeled corepressor peptides (NCOR1 ID2 peptide derived from NCOR1 gene; SMRT ID2 peptide derived from NCOR2 gene) in place of TRAP220.
(MEDI gene) coactivator peptide to convert from agonist mode (coactivator recruitment) into inverse-agonist mode (corepressor recruitment).

Proliferation assays
To enable cell-counting experiments, cell lines were transduced with a lentiviral vector encoding nuclear-targeted GFP, TagGFP2-H2B (Evrogen), and stable pools generated following selection for puromycin resistance. Cell lines were maintained for at least 7 days following selection prior to expansion and seeding into 96- or 384-well plates for further analysis. For kinetic proliferation assays, 96-well plates (n = 4 per condition) were imaged and counted every two hours using IncuCyte Zoom (Essen BioScience). Media and compounds were replaced approximately every 3–4 days. For endpoint assays to measure dose–response, cells were plated in 384-well plates, dosed with compound, and upon reaching approximately 70% to 90% confluence (IncuCyte Zoom) or incubated with CyQuant (Thermo Fisher) at the indicated time and plates were read with a fluorescent plate reader.

CRISPR/Cas9 genetic dependency studies
Cell lines were first transduced with a lentiviral vector encoding hSpCas9 under control of a tetracycline-inducible CMV promoter (CMV-TO; Thermo Fisher Scientific) and stable pools generated following selection for blasticidin resistance. Following confirmation of regulated Cas9 expression by Western blot analysis, the cells were transduced with a lentiviral vector encoding an sgRNA targeting the intronic sequence (sgPPARG-fl; CFP-expressing vector) of PPARG and stable pools were generated following selection for puromycin resistance. In addition to providing for regulated sgRNA expression, these lentiviral vectors also constitutively express one of three nuclear-targeted fluorescent proteins to enable unambiguous identification of transduced cells in subsequent cell counting experiments: YFP-expressing vectors were used for sgRNAs targeting PPARG; GFP-expressing vectors were used for sgRNAs targeting non-essential control genes, and RFP-expressing vectors were used for sgRNAs targeting essential control genes. This approach is described in more detail elsewhere (Stratdhe and colleagues, manuscript in preparation).

We evaluated 6–8 different sgRNAs per gene of interest using Western blot analysis to identify 2–3 highly active, doxycycline-inducible guides targeting PPARG (sgPPARG-3: GCTTCTCTCAGATAATAGGG, sgPPARG-6: GTTCTCAGAAATGGCCCTTGCA) for use in our experiments. We used KIF11 (sgKIF11-3: GGTGTTGGTCAGATGCGAGGT) as an essential control gene, and an sgRNA targeting PPARG intronic sequence (sgPPARG-21: GATACGTCGCAATTAGACAGG) was used as a nonessential control.

Following generation of stable pools of for each sgRNA were combined in equivalent numbers within replicate wells of 6-well plate and doxycycline was added to one of the replicates to induce Cas9 and sgRNA. Cells were passaged every 3–5 days and one replicate maintained under doxycycline induction, with second replicate maintained in the absence of doxycycline. During each passage, four replicate wells were passaged into 96-well plates for fluorescent imaging and fixed with methanol for imaging 1–3 days after passage into 96-well plates. Changes in relative abundance of cells containing the on-test sgRNA, nonessential sgRNA, and essential sgRNA were thus followed by comparing relative abundance of cells based on fluorescent label (yellow, cyan, red) through serial passages for a period of 28 days. Cells with stably transduced TREX-inducible vectors were all maintained continuously in MEM supplemented containing 10% FBS (Clontech).

Data availability

The provisional TCGA muscle-invasive urothelial carcinoma data are available from the Broad Institute TCGA Genome Data Analysis Center. Analysis-ready standardized TCGA data from Broad GDAC Firehose 2016_01_28 run. https://doi.org/10.7908/C11G0KM9. CCLE Affymetrix U133+2 arrays mRNA expression data is available through https://portals.broadinstitute.org/ccle/ (26). RNA sequencing data are available through the National Center for Biotechnology Information BioProject accession no. PRIRNA396067.

Results
PPARG signaling is activated by ectopic expression of RXRA p.S427F/Y and PPARG p.T447M-mutant alleles
To assess the biological effects of mutations in RXRA and PPARG, we ectopically expressed cDNAs encoding wild-type and mutant alleles of each gene in the SW780 bladder cancer cell line, which is wild type for RXRA and PPARG (26). RXRA hotspot mutant alleles p.S427F and p.S427Y, as well as a PPARG-mutant allele found in RT4 cells, p.T447M in PPARG isoform 1, NP_005028, as commonly referred to as PPARG p.T475M in isoform 2, NP_056953, were selected for initial testing. We performed RNA sequencing on parental SW780 cells in comparison with cells expressing wild-type or mutant PPARG or RXRA alleles. SW780 cells with ectopic expression of RXRA p.S427F/Y and PPARG p.T447M-mutant alleles demonstrated upregulation of canonical PPARG and PPARG target genes such as ACSL5, HMGCS2, and FABP4 (Fig. 1A). Immunoblot assays confirmed significant upregulation of several of the corresponding proteins in SW780 cells expressing mutant RXRA and PPARG alleles (Fig. 1B) even though parental SW780 cells appear to have elevated expression of PPARG target genes when compared with other bladder cancer cell lines (Fig. 1C). These data are consistent with the observations of increased expression of these PPARG targets in patient bladder tumors bearing RXRA hotspot mutations (9).

We also noted a discrete set of genes upregulated by RXRA p.S427F/Y, such as HMGSC2 and CEACAM5, which were minimally regulated by any of the PPARG alleles. In follow-up immunoblot studies, we included wild-type PPARG and observed that both HMGCS2 and CEACAM5 were selectively upregulated upon ectopic expression of PPARG, but not PPARG (Fig. 1B), indicating that these are likely PPARG targets. We also observed more pronounced effects of RXRA p.S427F compared with the p.S427Y allele on gene expression of HMGCS2, FABP4, and others by RNA sequencing (Fig. 1A), confirmed on the protein level by immunoblot assays (Fig. 1B). This may indicate a stronger phenotype for RXRA p.S427F, which is consistent with the higher frequency of this allele in bladder cancer patients (9). Ectopic expression of other RXRA-mutant alleles, selected on the basis of
apparent clusters (e.g., p.P231L, p.E233K), and sequence proximity to S427 (p.R421C, p.R421H, p.R426H, p.G429Y) from pancancer genomics analysis (29), had no activity in these assays (Supplementary Fig. S2).

**PPARG inverse-agonists decrease target gene expression in PPARG-activated cell lines**

The study of the phenotype of PPARG-activating genome alterations is facilitated by a wide variety of compounds that modulate PPARG activity, including agonists, antagonists, and inverse agonists. PPARG agonists increase recruitment of coactivators such as CREBBP, PGC1α (PPARGC1A), and MED1 to the RXRA-PPARG complex, leading to increased expression of target genes (Supplementary Fig. S3, top; ref. 30). PPARG inverse-agonists recruit co-repressors such as NCOR1, NCOR2, and HDAC3, leading to a decrease in basal expression of target genes (Supplementary Fig. S3, bottom; ref. 30). In contrast, PPARG antagonists have minimal effects on basal receptor function but are able to prevent both agonists and inverse-agonists from binding the receptor, thereby blocking their effects (Supplementary Fig. S3; ref. 30).

To evaluate the pharmacologic inhibition of PPARG in bladder cancer cell lines, we tested the impact of known PPARG modulating compounds in gene expression assays. In preliminary experiments across a panel of PPARG-activated bladder cancer cell lines (Fig. 1C), we found that dosing bladder cancer cell lines with T0070907, a PPARG inverse-agonist (31), but not GW9662, a PPARG antagonist (32), was able to reduce expression of the...
canonical PPARG target gene, FABP4 (Supplementary Fig. S4). Although these compounds have similar potency and selectivity profiles and share a remarkably similar chemical structure (Supplementary Table S1), they behaved quite differently in cells.

We engineered an FABP4 reporter cell line to enable higher throughput assays with which to evaluate PPARG transactivation. Briefly, the NanoLuc luciferase reporter gene was inserted into the 3'-UTR of the FABP4 gene in the PPARG-activated RT112/84 bladder cancer cell line using CRISPR/Cas9 mediated homology-directed repair (Supplementary Fig. S5). Because many compounds that target nuclear receptors are selective modulators, which have context-dependent activity profiles (30), we used this reporter cell line to characterize the activity of 13 previously characterized PPARG agonists, antagonists, and inverse-agonists (18, 25, 31–40). The full PPARG agonists, including rosiglitazone, pioglitazone, tesaglitazar, and GW1929, were able to increase the basal activity of the PPARG reporter from 5.5- to 7.4-fold in this assay (Fig. 2A, indicated in red). The partial agonists, including UVI3003 and SR1664, increased the basal activity of the reporter from 1.4- to 3.1-fold (Fig. 2A, indicated in black). The antagonists, including BADGE, SR202, GW9662, and SR2595, had minimal detectable effect on this unstimulated reporter (Fig. 2A, indicated in light blue). The PPARA-selective inverse agonist GW6471 exerted a modest inhibitory effect. Finally, the two inverse-

Figure 2.
PPARG inverse-agonists decrease basal activity by inducing active repression. PPARG-activated RT112/84 bladder cancer cell line engineered to contain the NanoLuc gene in the 3' UTR of a canonical PPARG target gene, FABP4, was used to profile the effect of compounds on PPARG-dependent gene transactivation. A, Dose–response testing of panel of PPARG modulators in the RT112/84 FABP4-NLucP reporter assay under unstimulated conditions to evaluate activity of agonists and inverse-agonists. B, Antagonist mode dose–response testing of panel of PPARG modulators in the RT112/84 FABP4-NLucP reporter assay performed by combined dosing of the PPARG agonist, rosiglitazone at the EC50, 20 nmol/L, in combination with a dose-response for test compounds, as indicated.
agonists tested, T0070907 and SR10221, reduced basal PPARG reporter activity by 85% to 88% (Fig. 2A, indicated in dark blue and green). Interestingly, the closely related structural analogs (Supplementary Table S1) of these compounds, GW9662 and SR2595, respectively, were essentially neutral antagonists in this assay.

To evaluate antagonist and inverse-agonist activity in more detail, compounds were tested in the presence of a PPARG agonist, rosiglitazone, for their impact on ligand-activated PPARG reporter activity. Here, the agonists gave little additional stimulation (Fig. 2B, indicated in black). The antagonists GW9662 and SR2595 decreased the agonist-induced signal back to the original baseline (Fig. 2B, indicated in light blue), whereas the inverse-agonists T0070907 and SR10221 further reduced the agonist-induced signal 80%–90% below baseline (Fig. 2B, indicated in red). The antagonists GW9662 and SR2595 (Fig. 3B, indicated in dark blue), increased signal 3- to 6-fold, whereas antagonists GW9662 and SR2595 (Fig. 3B, indicated in light blue), induced only a small increase in signal of 1.7-fold. A decrease in signal of 50%–60% was observed with the rosiglitazone, tesaglitazar, and GW9129 agonists (Fig. 3B, indicated in red).

In addition to the PPARG–NCOR2 interaction assay described above, we measured interactions between PPARG and a peptide from the corepressor NCOR1. In the PPARG–NCOR1 interaction assay, T0070907 induced signal 6- to 8-fold, whereas the antagonist GW9662 induced signal 2.5-fold and inverse agonist, SR10221, induced signal less than 2-fold, and antagonist, SR2595, had no effect (data not shown). The potent inverse agonist activity of SR10221 in cellular assays (Fig. 2; Supplementary Figs. S6 and S7) and biochemical PPARG–LBD–NCOR2 assay (Fig. 3B), but not PPARG–LBD–NCOR1 interaction assay (Fig. 3B), suggests that NCOR2 may be the functional corepressor mediating PPARG inverse-agonist activity in bladder cancer cells.

PPARG inverse-agonists inhibit the proliferation of PPARG-activated bladder cancer cells.

To determine whether PPARG modulators affect the proliferation and/or viability of PPARG-activated bladder cancer cells, we performed a direct cell counting–based assay. This assay likely avoids potential artifacts associated with the use of an ATP content–based assay, given that PPAR modulators are known to regulate cellular metabolic activity.

Both inverse-agonists, T0070907 and SR10221, significantly reduced proliferation of UM-UC-9 cells, compared with DMSO control (P < 0.001; Fig. 4A, indicated in green), with calculated IC_{50} values of 39 and 16 nmol/L. In contrast, both antagonists tested, GW9662 and SR2595, had no significant effects on cell proliferation (Fig. 4A, indicated in gray). These IC_{50} values align well with the calculated IC_{50}S from both the cell-based reporter gene assay (Fig. 2A) and the biochemical coactivator assay (Fig. 3B). They are also close to the respective reported biochemical IC_{50}S against PPARG, and two orders of magnitude below that reported for activity against PPARA and PPARD (31), suggesting that the observed antiproliferative effects in UM-UC-9 cells are due to downregulation of PPARG target genes.

We expanded this assay to include an additional 8 representative bladder cancer cell lines, including cell lines with PPARG amplification, RXRA p.S427F mutation, activated gene signature, and control cell lines with low level expression of PPARG and target genes. As we could not always accurately calculate an IC_{50} value, we used an alternative quantitative endpoint assay, which measures the relative number of cells in the DMSO vehicle control compared with treatment with 100 nmol/L of each compound with analysis performed at the time required for the cells to reach 50% confluency in the DMSO control. Representative data for the UM-UC-9 cell line are shown in Fig. 4B, and is tabulated for
the tested bladder cancer cell lines in Table 1. Similar to the full
dose–response assay (Fig. 4A), the T0070907 and SR10221
inverse-agonists reduce proliferation by 81% and 80% relative
to control, whereas the GW9662 and SR2595 antagonists have no
effect. This significant \( P < 0.01 \) preferential sensitivity to PPARG
inverse-agonists, but not antagonists, is maintained across all
of the PPARG-activated cell lines in the panel, including the RXRA
p.S427F cell line, HT-1197 (41), the PPARG-amplified cell lines,
5637 and UM-UC-9, and the PPARG activation gene signature cell
lines Cal29, UM-UC-3, and KU19.19 (Table 1). The SW1710,
UM-UC-3, and KU19.19 cells lines that did not exhibit high
expression of PPARG or target genes (Fig. 1C) were insensitive
to PPARG inverse-agonists and antagonists (Table 1). We
obtained similar results with these cell lines in parallel studies
using clonogenic assays to quantify colony-forming ability (Sup-
plementary Fig. S8). These data reveal that proliferation of the
PPARG-activated subset of bladder cancer cell lines, but not
control bladder cancer cell lines, is dependent on PPARG activity,
and inhibition of this activity with the inverse-agonists, T0070907
or SR10221, results in decreased proliferative potential.

PPARG-activated cell lines are genetically dependent on PPARG
To test for PPARG dependency in PPARG-activated cell
lines with an orthogonal approach, we performed CRISPR/Cas9

Figure 3. PPARG inverse-agonists induce a repressive complex by blocking interactions with coactivators and
inducing interactions with corepressor NCOR2. A TR-FRET assay was used to evaluate the ligand-dependent
interactions between Terbium-labeled PPARG ligand binding domain (PPARG-LBD) and fluorescein-labeled
peptides derived from nuclear receptor coregulators, as indicated. A, Dose-response testing of a panel of
PPARG modulators in an agonist biochemical model evaluating a PPARG-LBD - TRAP220 (MEDI)
coactivator peptide interaction assay to measure agonist-induced interactions, or inverse-agonist
induced decrease in interactions. B, Dose-response testing of a panel of PPARG modulators in an inverse-
agonist biochemical model evaluating PPARG LBD-NCOR2 (NCOR2) corepressor interactions.
knockout studies using a high-precision multicolor competition dependency assay (Strathdee and colleagues, manuscript in preparation). Briefly, we first validated that guide RNAs against PPARG were able to significantly diminish PPARG expression by Western immunoblot after normalization to loading control vinculin, VCL (Fig. 5A and B). We next compared the relative effect of knockout of PPARG on cell proliferation to knockout of an essential gene (KIF11) and to knockout of a nonessential gene (HPRT or PPIB) or PPARG intron control. The sgRNAs targeting these genes were cloned into lentiviral vectors that coexpress one of three different fluorescent proteins, YFP, RFP, or CFP, and which allowed for unambiguous identification of transduced cells in complex pools: PPARG knockout cells were labeled with YFP, essential control knockout cells were labeled with RFP, and nonessential (or PPARG intron) control knockout cells were labeled with CFP. In the competition format, replicate pools of cells are generated at the beginning of the experiment in which each gene knockout/color are at equivalent abundance. Changes in relative abundance of each population are monitored during progressive serial passage by counting fluorescent nuclei using high-content imaging. Relative changes are plotted as percent of control of the normalized prevalence of on-test sgRNAs as a percent of nonessential sgRNA control targeting an intron of PPARG.

Analysis of three bladder cancer cell lines showed a clear PPARG dependency in PPARG-activated cells. The growth of the SW1710 cell line, which shows low PPARG/FABP4 expression (Fig. 1C), is insensitive to PPARG knockout (Fig. 5C). In contrast, HT-1197 the only known cell line with an RXRA p.S427F mutation (41), exhibited a strong PPARG dependency in this CRISPR competition assay (Fig. 5D). The Cal29 cell line, with a highly activated PPARG signaling pathway (Fig. 1), also exhibits clear PPARG dependency (Fig. 5E). In preliminary experiments we determined that the RT4 cell line supported inadequate hCas9 expression for use in gene knockout experiments, which is unfortunate since it is the only cell line available with a PPARG p.T447M mutation. Note that we did not include any cell lines containing PPARG focal amplifications to avoid the well-known copy number sensitivity artifact inherent to the CRISPR/Cas9 platform (42, 43). We conclude that from the above analyses that PPARG-activated bladder cancer cell lines are dependent upon a functional PPARG.

**Discussion**

Recently, genomic analysis of bladder cancer revealed that the PPARG signaling pathway is significantly activated in tumors, and that this can be driven by either RXRA p.S427F/Y mutations or...

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**Table 1.** Relative proliferation rate of bladder cancer cell lines treated with PPARG modulators.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pathway alteration</th>
<th>GW9662</th>
<th>SR2595</th>
<th>TO070907</th>
<th>SR10221</th>
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<tr>
<td>HT-1197</td>
<td>RXRA p.S427F</td>
<td>127</td>
<td>78</td>
<td>48*</td>
<td>64*</td>
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<td>5637</td>
<td>PPARG amplified</td>
<td>93</td>
<td>117</td>
<td>51*</td>
<td>73*</td>
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<td>19*</td>
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<td>101</td>
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NOTE: Bladder cancer cell lines were treated with vehicle or GW9662, SR2595, SR10221, or TO070907 at 100 nmol/L in long-term kinetic proliferation assays lasting 6–12 days. Data reported as percent of DMSO control, calculated by determining the relative number of cells for treatment versus DMSO control at the time point where DMSO control reached 50% confluence (see Fig. 4B for graphical representation) by counting fluorescently labeled nuclei (IncuCyte Zoom). Significant differences in cell number for test sample relative to DMSO control were calculated using two-way ANOVA with Dunnett multiple comparison test.

\*P < 0.001.
\*P < 0.01.
Figure 5.

Cell lines with PPARG activation are dependent on PPARG. A, PPARG sgRNA’s 3 and 6 knockout PPARG protein in SW637 cells. A, Western immunoblot of PPARG and loading control VCL. B, Quantification of PPARG Western blot signal after normalization to VCL, reported as ratio to VCL, and expressed in percent of control (POC), normalized to VCL. Data represent mean ± SD.

Panel C, D, E represents competition screen to measure relative proliferation of cells harboring sgRNA targeting PPARG (yellow), nonessential control of PPARG intron (cyan), and essential control gene KIF11 (red). Cells lines were infected with lentivirus encoding both fluorescent marker and sgRNA prior to pooling cells for assay. C, SW1710 (not altered); D, HT-1197 (RXRA p.S427F); and E, Cal 29 (PPARG-activated). Data are represented as percent of control (POC), normalized prevalence of test sgRNAs as a percent of the non-essential PPARG intron control sgRNA (mean ± SD).

PPARG focal amplifications (9, 15). Here we confirm that these alterations, in addition to PPARG p.T447M mutation, which may be emerging as a new hotspot (Supplementary Fig. S10), activate the PPARG signaling pathway, and that cell lines with the corresponding mutations are genetically dependent on PPARG and are also sensitive to pharmacologic inactivation using PPARG inverse-agonists.

One model to explain this data is that these alterations confer ligand-independent activation of PPARG. In the case of RXRA p.S427F/Y and PPARG p.T447M mutations this could be achieved by the gain of hydrophobic interactions that lock PPARG helix 12 into the active conformation, phenocopying the agonist-induced state in the absence of ligand. Co-crystal structures of RXRA/PPARG (e.g., PDB ID: 1FM6 and PDB ID: 5JI0; refs. 15, 44) show that the S427 position of RXRA is located in the dimerization interface with PPARG in the ligand-activated state, directly adjacent to both the T447 residue and c-terminus Y477 residue of PPARG. This positioning is also conserved in homology models of RXRA/PPARA (45). RXRA p.S427F/Y mutations may also disrupt interactions between RXRA and its other heterodimer partners (e.g., RARA, VDR, TR), further shifting equilibrium of RXRA further toward PPARs (15).

It has been established that other mechanisms can lead to ligand-independent activation of PPARG. For example, signaling by insulin through the actions of MAP kinases leads to phosphorylation of PPARG in the AF-1 domain, which can lead to ligand-independent activation (46). Insulin-dependent PPARG activation is not sensitive to inhibition by the PPARG antagonist GW9662, whereas ligand-driven activation by PPARG agonist, ciglitazone, is sensitive to GW9662 (47). Here we show that PPARG-activated bladder cancer cells, through PPARG amplification, RXRA p.S427F mutation, or other unknown mechanisms, are similarly not responsive to antagonists, including GW9662 and SR2595, but are sensitive to inverse-agonists. Because PPARG inverse-agonists induce a conformational change in the ligand-binding domain to actively recruit corepressors to the complex, these could overcome ligand-independent signaling, as our studies show.

The impact of PPARG on bladder cancer signaling is supported by the evidence that PPARA/PPARG dual agonists cause bladder cancer in rodents, although there are conflicting epidemiological data that PPARA/PPARG agonists are associated with increased rates of disease in humans (20, 22–24, 48).

PPARG as a therapeutic target in bladder cancer can be seen as analogous to targeting androgen receptor in prostate cancer or estrogen receptor in breast cancer. One of the hallmarks of luminal cancers is the expression of a ligand-activated nuclear receptor. Therapeutic targeting of the nuclear receptors in patients with these cancers can be a very effective therapeutic approach as in the example of targeting ESR1-positive luminal breast cancer with anti-estrogens and AR-positive prostate cancer with androgen deprivation therapy. PPARG agonists upregulate expression of luminal differentiation markers LIPK1A, LIPK1B, and KRT20 in primary rat urothelial cells (49). These same genes, plus GATA3, and FOXA1 are the key luminal markers of bladder cancer from human patients (13, 14) and bladder cancer cell lines (16). The lineage-defining role of GATA3, FOXA1, and PPARG in luminal bladder cancer is reminiscent of luminal breast cancer, in which coordinated expression of GATA3, FOXA1, and ESR1 enable chromatin remodeling and regulate luminal gene expression programs (50, 51); whereas in prostate cancer, FOXA1 and GATA2...
(52) coordinate regulatory activity (53) and distribution and selectivity for AR response elements.

The steroid hormone receptors ESR1 and AR are also in the nuclear receptor superfamily; however, distinct from PPARG, they utilize high-affinity ligands for signaling. Endogenous production of estrogen in breast tissue, and testosterone/dihydrotestosterone in the testes, are required for receptor activation. Therefore, in the context of ligand-activation, antagonists of ESR1 and AR are effective therapies for primary cancers. Another effective strategy in breast and prostate cancer are therapies leading to inhibition of ligand production, such as the use of aromatase inhibitors for treatment of breast cancer and androgen deprivation therapy for prostate cancer. In contrast, PPARG does not have a high-affinity endogenous ligand and is considered a lipid sensor, with low affinity for its ligands (54). Therefore, mechanisms leading to ligand-independent activation of PPARG, and not ligand-dependent signaling, appear to be the primary driver of PPARG activity and will require different pharmacologic properties than in the case of targeting ligand-activated ESR1 or AR with a pure antagonist.

Patients treated with anti-estrogen and anti-androgen therapy commonly develop resistance, with common mechanisms being somatic alterations that lead to either ligand hypersensitivity or ligand-independent signaling. In the case of ESR1, recurrent mutations at Y537 lead to ligand-independent signaling (Robinson and colleagues, 2013) and due to being in the ligand-binding domain, also confer resistance to ESR1 antagonists. In the case of AR, gene amplification leads to ligand hypersensitivity, whereas point mutations and alternative splicing can lead to ligand-independent activation (55). To attenuate ligand-independent activation of ESR1, a new class of compounds was developed that lead to receptor degradation. Selective estrogen receptor degraders have had promising clinical outcomes, with fulvestrant reaching clinical approval and providing a new hope for ER+ breast cancer patients refractory to selective estrogen receptor modulators and aromatase inhibitors. On the basis of the fact that there appears to be high level of ligand-independent PPARG signaling in bladder cancer, we focused efforts on validating inverse-agonists as a candidate therapeutic strategy. However, a selective PPARG destabilizer could be another promising therapeutic approach worth exploring.

We have demonstrated a genetic and pharmacologic dependence on PPARG in PPARG-activated, luminal bladder cancer cell lines. Our studies provide a well-defined patient population and clear therapeutic hypothesis. Because PPARG agonists rosiglitazone and pioglitazone are used for the treatment of diabetes by a mechanism of sensitizing cells to insulin, lowering blood glucose, and lowering lipid levels (56), one may predict that a PPARG inverse-agonist may have an opposing effect, eliciting symptoms of diabetes. Furthermore, patients with deleterious PPARG mutations have an increased risk for diabetes and can exhibit lipodystrophy and insulin resistance (57). Although there are concerns for on-mechanism complications for PPARG inverse-agonists as a potential therapy for bladder cancer, there is renewed hope that rigorous testing and a deep understanding of emerging PPARG biology and pharmacology (25) can overcome these hurdles through selective receptor modulation. Recent advances have highlighted a potential role for oncogenic activation of PPARG in bladder cancer to regulate inflammatory cytokines, thereby regulating immune cell infiltration and immunosurveillance (15). Further studies of PPARG in bladder cancer will help to evaluate whether PPARG inverse agonists can complement conventional and emerging therapies including other genomically defined therapeutic targets such as FGFR inhibitors, in addition to immune checkpoint blockade.

Disclosure of Potential Conflicts of Interest

J.T. Goldstein reports receiving a commercial research grant from Bayer AG. A.C. Berger reports receiving other commercial research support from Bayer AG. F.F. Duke reports receiving a commercial research grant from Bayer AG. L. Furst reports receiving a commercial research grant from Bayer AG. A.D. Cherniack reports receiving a commercial research grant from Bayer AG. M. Meyerson reports receiving a commercial research grant from Bayer AG, has ownership interest in Origimed; and also is a consultant/advisory board member for Origimed. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: J.T. Goldstein, C.A. Strathdee

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.T. Goldstein, A.C. Berger, J. Shih, A.D. Cherniack, C.A. Strathdee

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