A small-molecule inhibitor of Tcf/β-catenin signaling down-regulates PPARγ and PPARδ activities

Shlomo Handeli and Julian A. Simon

Clinical Research and Human Biology Divisions, Fred Hutchinson Cancer Research Center, Seattle, Washington

Abstract

Activation of the Wnt/β-catenin signaling pathway occurs in several types of cancers and thus it is an attractive target for anticancer drug development. To identify compounds that inhibit this pathway, we screened a chemical library using a cell-based β-catenin/Tcf–responsive reporter. We identified FH535, a compound that suppresses both Wnt/β-catenin and peroxisome proliferator–activated receptor (PPAR) signaling. FH535 antagonizes both PPARγ and PPARδ ligand–dependent activation and shows structural similarity to GW9662, a known PPARγ antagonist. The effect of FH535 on β-catenin/Tcf activity is reduced in cells carrying a deletion of the *PPARδ* gene, as well as by the PPARγ agonist lysophosphatidic acid. Mechanistically, FH535 inhibits recruitment of the coactivators β-catenin and GRIP1 but not the corepressors NCoR and SMRT. Its repression of β-catenin recruitment, in comparison with GW9662, is linked to FH535's unique capability to inhibit the Wnt/β-catenin signaling pathway. The antiproliferation effect of the compound observed on some transformed colon lung and liver cell lines is suggestive of its potential therapeutic value in the treatment of cancer. [Mol Cancer Ther 2008;7(3):521–9]

Introduction

The Wnt signaling pathway is important in normal development and in cancer (reviewed in refs. 1, 2). This signaling pathway is regulated by Wnt ligands, the APC-Axin complex and β-catenin. In development, signaling that stabilizes β-catenin is mainly mediated by the Wnt ligands. However, in human cancers such as hepatocellular and colorectal tumors, β-catenin stabilization is often the result of mutation in the tumor suppressor genes *Axin* and *APC*, or in the proto-oncogene *β-catenin*. β-Catenin stabilization leads to its accumulation and subsequent translocation to the nucleus, where it forms complexes with transcription factors of the Tcf/Lef family. The transcriptionally active β-catenin/Tcf complex exerts its cell proliferation and tumorigenic effects by promoting the transcription of growth controlling genes like *c-Myc* and *cyclin D1*. Wnt/β-catenin signaling pathway plays a central role in regulating the balance between stem cell growth and differentiation. Thus, the degree of Wnt signaling activation is an important modulator of the stem cells cancerous potential. In colorectal cancer, the necessary initiating APC or β-catenin mutations are not sufficient for maximum Wnt activation. Other intrinsic somatic mutations such as the oncogene *Ras*, as well as extrinsic factors like prostaglandins, are likely to play a rate-limiting role in Wnt signaling activation and contribute to the cancer development from the initial stem cell transformation to the metastasis stage (3). Increasing evidence shows that suppression of Wnt signaling can be achieved by targeting pathways that cross-talk with the Wnt signaling pathway. For example, both cyclooxygenase-2 and integrin-linked kinase small-molecule inhibitors attenuate β-catenin/Tcf–dependent transcription in colorectal cancer cells harboring mutated Wnt signaling (reviewed in ref. 4).

Peroxisome proliferator–activated receptors (PPAR) are members of the nuclear hormone receptor superfamily. The ligand-activated transcription by these receptors requires heterodimerization with retinoid X receptor, interaction with different coactivators as well as binding to PPAR-response elements (PPRE). There are three PPAR isotypes (α, γ, and δ), differing in their tissue distribution, physiologic functions, and ligand specificity. Fatty acids and their derivatives are natural PPAR agonists. Synthetic agonists have been reported for all PPAR isoforms, whereas antagonists have been identified only for PPARγ.

The physical interaction between β-catenin and PPARγ suggests a possible mechanism of cross-talk between the Wnt and the PPAR signaling pathways. On the transcriptional level, β-catenin enhances PPARγ activity whereas PPARδ is a target for β-catenin/Tcf regulation (5, 6). A clear interaction between these pathways is observed during adipogenesis. Adipogenic differentiation is regulated by reciprocal inhibitory signals between PPARγ and Wnt ligands; Wnt1 and Wnt10 promote the growth of preadipocytes whereas PPARγ agonists repress the Wnt/β-catenin signaling and advance their differentiation (7).

The contribution of PPARγ and PPARδ to Wnt/β-catenin–induced carcinogenesis remains unclear, as there are genetic and pharmacologic data suggesting that PPARs either promote or inhibit colon cancer (reviewed in refs. 8, 9). For example, treatment of Apc^{min} mice, which are predisposed to intestinal polyposis with the PPARγ agonist troglitazone, enhanced colon polyp development (10, 11), whereas treatment with pioglitazone, another PPARγ

Received 9/12/07; revised 10/31/07; accepted 12/11/07.

Grant support: Clinical Research Division, Fred Hutchinson Cancer Research Center (J.A. Simon).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Julian A. Simon, Fred Hutchinson Cancer Research Center, D2-100, 1100 Fairview Avenue North, Seattle, WA 98109. Phone: 206-667-6241; Fax: 206-667-5255. E-mail: jsimon@fhcrc.org

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-07-2063

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

agonist, suppressed polyp formation (12). Similarly, conflicting results were observed using the PPARδ agonists GW501516 and GW0742 (13, 14).

Another class of compounds that modulate both Wnt/β-catenin and PPAR activity is the nonsteroidal anti-inflammatory drugs (NSAID). The pharmacologic action of NSAIDs has been attributed to their inhibition of cyclooxygenase activity. However, a large number of studies have suggested that the anticarcinogenic efficacy of NSAIDs is independent of their cyclooxygenase inhibition. Several NSAIDs, including indomethacin, weakly interact with PPARγ and stimulate its activity (15, 16). Indomethacin and other NSAIDs were also reported to suppress PPARδ activity (6, 17). The role of PPAR regulation to the overall ability of NSAIDs to repress the Wnt/β-catenin pathway is not fully understood.

Here, we report the identification of a low molecular weight compound (FH535) that suppresses β-catenin/Tcf–mediated transcription. FH535 behaves as a dual PPARγ and PPARδ antagonist that is able to inhibit GRIP1 and β-catenin recruitment. Comparisons between FH535 and the PPARγ antagonist GW9662, which allows β-catenin recruitment, suggest that inhibition of the Wnt/β-catenin pathway may require modulation of the interaction between PPARs and β-catenin.

Materials and Methods

Reagents

The chemical library used for the screen is part of the DIVERSet collection from ChemBridge. ChemBridge was also the source for FH535 and its analogues. L165041 was purchased from Calbiochem-EMD Biosciences. All other PPAR ligands and nitric oxide–donating aspirin were obtained from Cayman Chemical.

High-throughput Library Screen

Three copies of the optimized or mutated Tcf-binding element from TOPFLASH or FOPFLASH (18) driving a secreted alkaline phosphatase reporter gene were cloned into pCEP4 plasmid (Invitrogen), replacing the cytomegalovirus promoter. The plasmids were transfected into HepG2 cells, and hygromycin-resistant clones were pooled. Library screening was done at 20 μmol/L concentration in HepG2 serum-free media (19). Hits were tested in the HCT116 cell line for inhibition of TOPFLASH luciferase activity but not for inhibition of a reporter activity controlled from β-actin promoter (details of the reporter construct used in the screen and the stepwise characterization scheme for prioritization of hits is available in the Supplemental Materials).[1]

Transfection and Reporter Gene Assays

TransIT-LT1 transfection reagent (Mirus) was used according to the manufacturer's instructions. After transfection of reporter constructs (18–24 h), cells were trypsinized and replated in drug-containing assay plates for another 24 h. Alkaline phosphatase and luciferase activities were measured using CSPD Emerald-II (Tropix-Applied Biosystems) or steadylite-HTS (Perkin-Elmer), respectively. When an internal control for transfection efficiency was required, the pCMV/β-galactosidase vector was cotransfected with the luciferase reporter constructs and β-galactosidase activity was assayed with Galacto-Light Plus (Tropix-Applied Biosystems). All assays were done in triplicate using a 96-well plate format and a TopCount-NXT luminescence counter (Packard).

Plasmids

The optimized and mutated Tcf-binding element–driven luciferase reporters (TOPFLASH, FOPFLASH) were provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). The PPRE-driven luciferase reporter (ptk-PPRx3-luc; ref. 20) was a gift from Dr. R.M. Evans (Salk Institute, La Jolla, CA). The PPARγ protein fused to VP16 activation domain or the gal4 DNA-binding domain (pVP16-PPARγ2, pECE72gal4-PPARγLBD) and gal4-NCoR (pECE72gal4-NcoR) plasmids were made available by Dr. R.N. Cohen (University of Chicago, Chicago, IL). The gal4-GRIP plasmid (pSG424-mGRIP1; ref. 21) was a gift from Dr. F. Saatcioglu (University of Oslo, Oslo, Norway). Full-length mouse PPARδ coding sequence was amplified and fused in-frame downstream of VP16 activation domain in the pVP16 vector (Clontech) or downstream of the gal4 DNA-binding domain in the pSG424 vector (22). The PPARγC285A point mutation was introduced into the human *PPARγ* gene by replacing the *Msc*I-*Bsa*AI fragment with a PCR product amplified with the primers 5′-CCATCCGCATCTTTCAGGGCGCG-CAGTTTCG-3′ and 5′-GTGCTCTGTGACGATCTGCCT-GAGG-3′. The PPARδC248A point mutation was introduced into the mouse *PPARδ* gene by replacing the *Bst*XI-*Bsa*AI fragment with a fragment composed of 5′-GTGTTCTACCGGGCCCAGTCCACCACA-3′ and 5′-GTGGACTGGGCCCGGTAGAACAC-3′. The gal4/β-catenin fusion protein was constructed by cloning the gal4 DNA-binding domain upstream of the full-length human β–catenin gene in pCS2+ vector. The UAS–thymidine kinase reporter [p(UAS)5 tk-LUC] harbors five gal4-binding sites upstream of a minimal thymidine kinase promoter followed by the luciferase gene (a gift from S.J. Collins, Fred Hutchinson Cancer Research Center, Seattle, WA).

Cell Cultures

Cell lines were obtained from the American Type Culture Collection, except for the following cell lines: Huh7 was obtained from Dr. M. Katze (University of Washington, Seattle, WA). HCC15 and NCI-H1703 were a gift of Dr. A. Gazdar (UT Southwestern Medical Center, Dallas, TX). HCT116 PPARδ$^{+/+}$ WT and HCT116 PPARδ$^{-/-}$ KO1 (23) were generously provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). All cell lines were cultured at 37°C and 5% CO_2. The appropriate medium was supplemented with 10% fetal bovine serum (FBS), 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. In HCT116 serum-free

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

medium, the FBS was substituted with 10 μg/mL of insulin, 5.5 μg/mL of transferrin, 35 nmol/L of sodium selenite, and 10 ng/mL of human epidermal growth factor (Life Technologies-Invitrogen). Cell viability was determined by the modified ^{3}H-thymidine incorporation assay (24). Briefly, cells were plated in 96-well microplates for 24 h and treated in triplicate with various concentrations of the test compound. After 48 h of compound exposure, the cells were incubated for an additional 48 h in compound-free medium. The cells were then incubated in medium containing ^{3}H-thymidine for 24 h, washed and mixed with the scintillant in the 96-well plate. Individual wells were counted with a 96-well scintillation counter (TopCount, Packard Instruments) and the LC_{50} was calculated.

Reverse Transcription-PCR

Total RNAs were extracted from HCT116 cells by the RNeasy kit and cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen). The primer sequences for amplification were: GAPDH-F 5′-ATGATCTTGAGGCTGTTG-3′, GAPDH-R 5′-CTCAGA-CACCATGGGGAA-3′; TCF-4F 5′-TTCAAAGACGACGGC-GAACAG-3′, TCF-4R 5′-TTGCTGTACGTGATAAGAGG-CG-3′. PCR amplification was done using Taq Polymerase (Fisher) for 20 cycles at 50°C (GAPDH) and 57°C (TCF-4) annealing temperatures.

Results

FH535 Antagonizes β-Catenin/Tcf – Mediated Transcription

To identify inhibitors of the Wnt/β-catenin pathway, we modified the existing TOPFLASH reporter system (18) to suit a high-throughput screen. Episomal reporter constructs containing three copies of either an optimized or mutated Tcf-binding element were stably transfected into $HepG_2$ hepatocellular carcinoma cells expressing high levels of nuclear β-catenin. Robust reporter activity was detected in clones containing the optimized element, which was 10-fold higher than activity in clones expressing the mutated element (data not shown).

Using this cell-based reporter system, we screened a diverse library of 11,600 low molecular weight compounds for the inhibition of β-catenin/Tcf–mediated transcription. Initially, two structurally related compounds (FH525 and FH614; Fig. 1A) were found to be potent inhibitors. Thirteen additional analogues were tested and another two active compounds were identified (FH535 and FH610; Fig. 1A). The nitro group located at the para or meta position in all four compounds is critical for function. Substitution of this nitro group with any of six other functional groups (OH, ethyl, ethylcarbonate, benzamide, *O*-benzyl, *para*-tolylsulfonamide) abolishes activity. In four cell lines harboring deregulation of the Wnt/β-catenin pathway, FH535 was found to be the best β-catenin/Tcf inhibitor (Fig. 1C). FH535 and two additional compounds (FH610 and FH614) were more active than nitric oxide–donating aspirin, a NSAID that has been shown to disrupt the formation of the β-catenin/Tcf complex at the drug concentration used here (ref. 25; Fig. 1C).

FH535 Antagonizes both PPARγ and PPARδ Activity

The newly identified β-catenin/Tcf inhibitors also share structural similarity to the known PPARγ antagonists GW9662 and T0070907 (refs. 26, 27; Fig. 1B). All of the compounds contain the nitro group, differing mainly in the

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

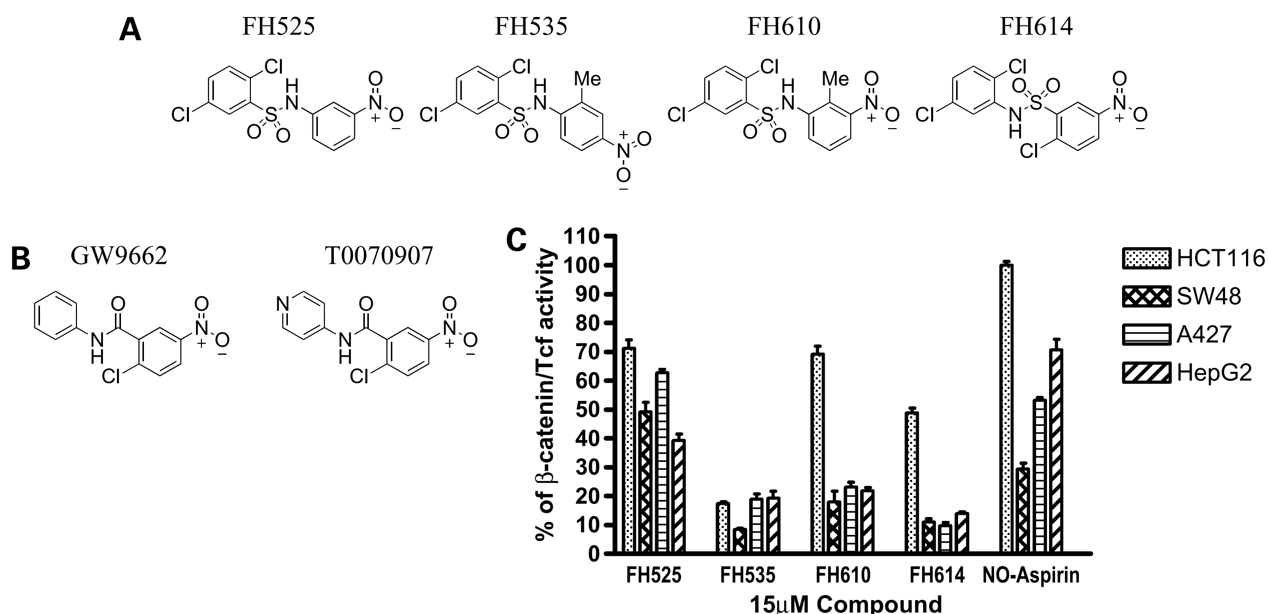

Figure 1. Inhibitors of β-catenin/Tcf – mediated transcription are structurally similar to PPARγ antagonists. **A,** chemical structures of β-catenin/Tcf – mediated transcription inhibitors. **B,** chemical structures of the PPARγ antagonists GW9662 and T0070907. **C,** inhibition of β-catenin/Tcf – dependent luciferase reporter activity in cell lines harboring stable β-catenin. Treatment with vehicle (DMSO) only was used to calculate the 100% activity for each cell line. FH535 is the most effective Wnt/β-catenin inhibitor.

central amide or sulfonamide groups as well as their orientation. Because PPARγ and PPARδ have been implicated in Wnt/β-catenin pathway regulation (reviewed in ref. 8), we tested the ability of our most active compound, FH535, to antagonize these PPARs.

First, we investigated the transactivation of a reporter construct containing three copies of the acyl-CoA oxidase PPAR-response element (20). In HCT116, this reporter is active and is sensitive to the addition of PPARγ and PPARδ agonists (Fig. 2). FH535 inhibits the reporter-dependent activity driven from natural PPAR ligands found in the cellular environment (Fig. 2A) as well as from the added ligands (Fig. 2B and C). A more direct assay for PPAR activity uses PPAR fused to the gal4 DNA-binding domain. Treatment with PPARγ or PPARδ agonists causes the activation of the UAS–thymidine kinase reporter (see Materials and Methods for details) in cells cotransfected with the relevant PPAR-gal4 chimera (Fig. 3A and B). This PPAR agonist–dependent transactivation is inhibited when FH535 is present.

We next evaluated the role of PPARδ in the inhibition of β-catenin/Tcf–mediated transcription by FH535. PPARδ-null HCT116 cells are more resistant to FH535 treatment than their matched paired cells expressing the wild-type PPARδ protein (Fig. 3C). However, deletion of PPARδ is not sufficient to fully counteract FH535 inhibition, suggesting that PPARδ is not the only target by which FH535 inhibits the Wnt/β-catenin pathway. Parallel to these findings, GW9662 is unable to inhibit β-catenin/Tcf–signaling regardless of PPARδ cellular levels (Fig. 3C).

FH535 Activity Does not Require PPARγ Cys285 and PPARδ Cys248

The PPARγ antagonists GW9662 and T0070907 irreversibly modify Cys^{285} in the PPARγ ligand–binding site via a nucleophilic aromatic substitution of chlorine (26, 27). The same cysteine residue is essential for the activity and covalent binding of some PPARγ agonists such as 15d-PGJ2 but not for rosiglitazone (28). Among our four active compounds, only FH614 has a chemical structure capable of cysteine residue arylation (Fig. 1C). This strongly suggests that the antagonistic activity of our compounds does not require the modification of a cysteine. However, because PPARγ Cys^{285} and its equivalent Cys^{248} in PPARδ may be important for a noncovalent interaction with the compounds, we mutated this cysteine to alanine and found that these changes had no significant bearing on FH535 antagonism (Fig. 3A and B). Thus, FH535 activity does not require the same PPARγ binding residues that GW9662 uses. The use of different PPAR residues for ligand binding was also observed for the PPARγ agonists, rosiglitazone and lysophosphatidic acid (29).

Both Serum and Lysophosphatidic Acid Reduce FH535 Inhibition of the Wnt/β-Catenin Pathway

We noticed that inhibition of PPRE-dependent activity in defined medium requires lower FH535 concentrations than in serum-containing medium (Fig. 2B and C). Because a variety of fatty acids and their metabolites are secreted into plasma and are naturally present in the serum, it is likely

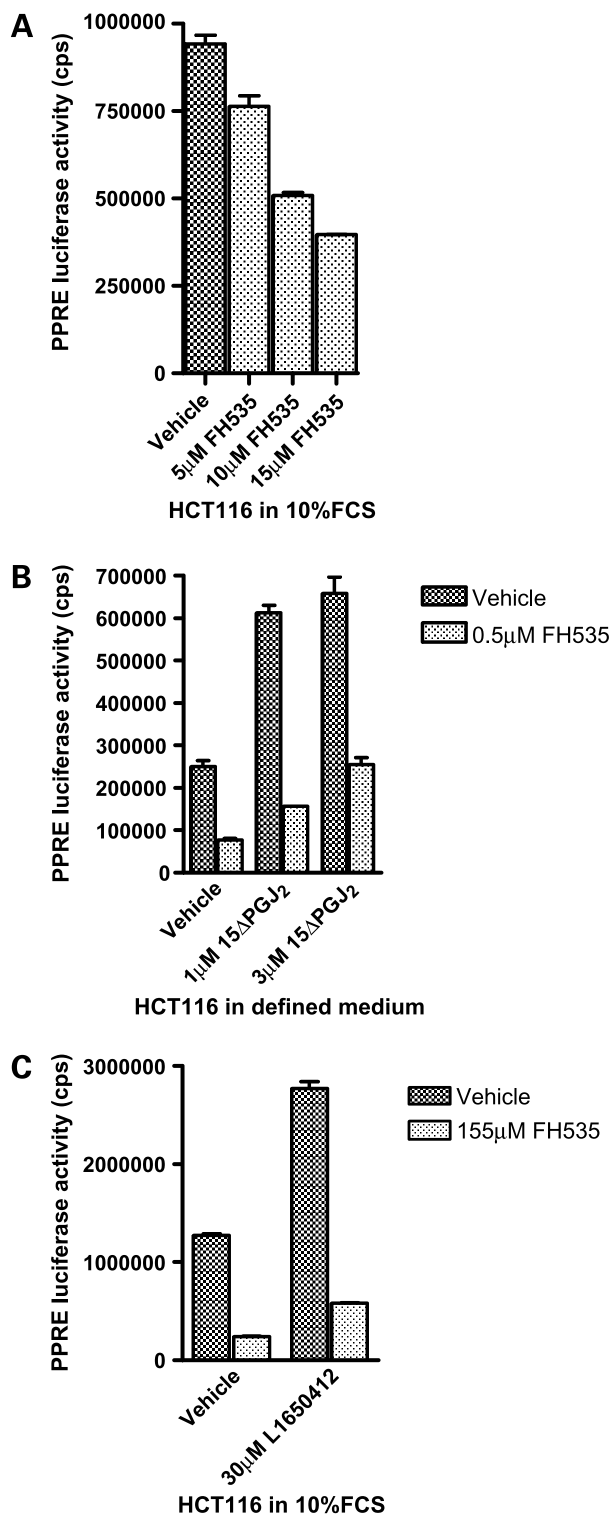

Figure 2. FH535 inhibits PPARγ and PPARδ transactivation in HCT116 cells. **A,** FH535 inhibits PPRE luciferase reporter activity. Cells were assayed in 10% FBS. **B,** FH535 inhibition of the PPRE reporter is suppressed by the PPARγ agonist 15ΔPGJ2. Cells were assayed in serum-free medium. **C,** FH535 inhibition of the PPRE reporter is suppressed by the PPARδ agonist L165041. Cells were assayed in 10% FBS.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

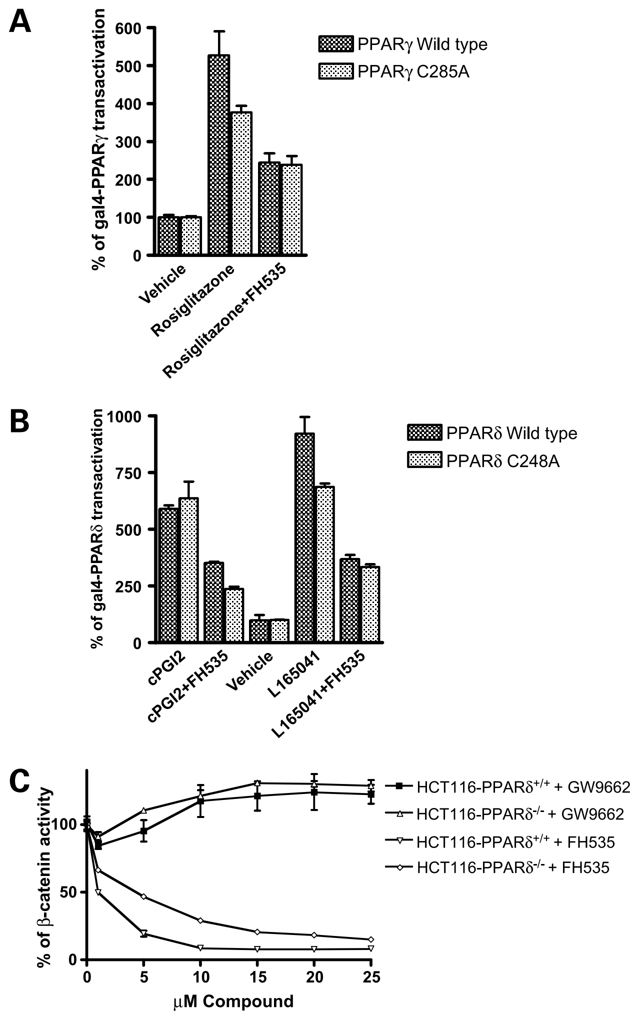

Figure 3. FH535 activities depend on functional PPARδ but do not require a cysteine residue in the PPAR ligand – binding domain. **A,** similar inhibition of gal4-PPARγ wild-type and C285A transactivation by FH535. Rosiglitazone (1 μmol/L) and FH535 (15 μmol/L) were used for PPARγ modulation in the HCT116 cell line. **B,** similar inhibition of gal4-PPARδ wild-type and C248A transactivation by FH535. cPGI2 (15 μmol/L) or L165041 (30 μmol/L) were used for PPARδ induction and FH535 (15 μmol/L) was used for PPARδ inhibition in the HCT116 cell line. **C,** inhibition of β-catenin/Tcf – dependent luciferase reporter activity in HCT116 cell lines containing wild-type PPARδ or homozygous deletion of PPARδ. FH535 inhibition of Wnt/β-catenin partially depends on the PPARδ, whereas GW9662 is unable to regulate the Wnt/β-catenin signaling.

that serum contains PPAR agonists capable of suppressing the effects of FH535. The opposing relationship between FH535 and PPAR agonists with regard to PPAR transactivation (Fig. 3A and B), raises the possibility that PPAR agonists can also counteract FH535 inhibition of the Wnt/β-catenin pathway. First, we tested the effect of serum on this FH535 activity. FH535 is five times more active in defined medium than medium containing serum (Fig. 4A). Adding albumin to the defined medium reduced FH535 activity only 2-fold (data not shown). Next, 10 natural and synthetic PPAR ligands representing a broad spectrum of binding affinities were tested. Of those examined, lysophosphatidic acid was the only ligand found to reduce the inhibition activity of FH535 on the β-catenin/Tcf reporter (Fig. 4B). Lysophosphatidic acid is a pleiotropic growth factor–like lipid that mediates its effects through the activation of G protein–coupled receptors LPA1-4 and PPARγ (30). Thus, lysophosphatidic acid could either directly interfere with FH535 inhibition of PPARγ or the activation of lysophosphatidic acid receptors could indirectly suppress the activity of FH535.

FH535 Inhibits β-Catenin and GRIP1 Recruitment to PPARγ and PPARδ

To investigate the mechanism in which FH535 suppresses β-catenin/Tcf and PPAR-dependent transactivation, we focused on β-catenin and the coactivator GRIP1 because

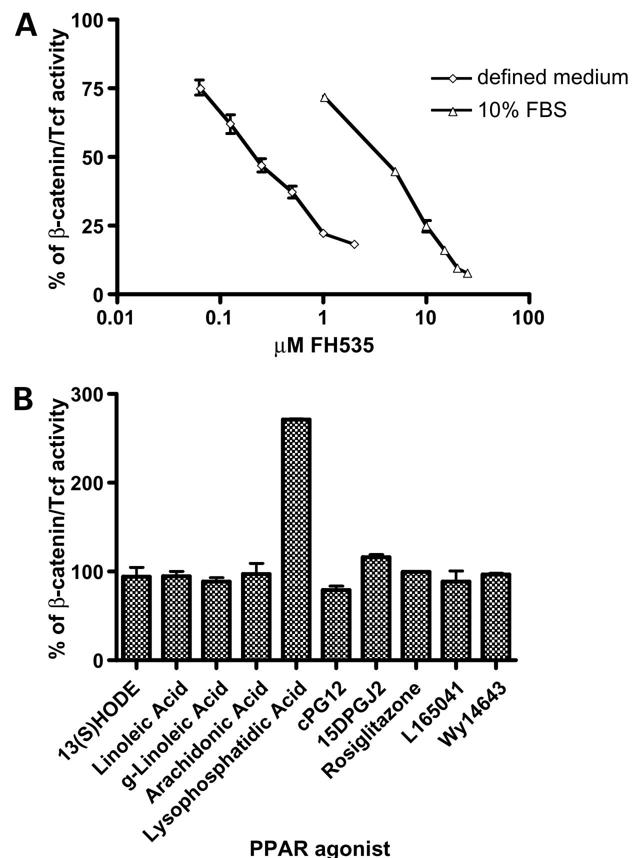

Figure 4. Serum and lysophosphatidic acid reduces FH535 inhibition of Wnt/β-catenin pathway. **A,** FH535 inhibition of β-catenin/Tcf – dependent luciferase reporter activity in HCT116 cells grown in defined medium or medium containing 10% FBS. Treatment with vehicle only was used to calculate the 100% activity. **B,** effects of PPAR agonists on FH535 inhibition of β-catenin/Tcf – dependent luciferase reporter activity in HCT116 cells. Treatment with FH535 alone reduces β-catenin/Tcf activity to ~20% of vehicle controls and was used as the 100% benchmark to evaluate the effect of FH535 and PPAR agonists. Concentration of PPAR agonists used: 10 μmol/L of 13(*S*)HODE, 15 μmol/L of linoleic acid, 15 μmol/L of γ-linoleic acid, 15 μmol/L of arachidonic acid, 25 μmol/L of lysophosphatidic acid, 15 μmol/L of cPGI2, 1 μmol/L of 15dΔPGJ2, 1 μmol/L of rosiglitazone, 30 μmol/L of L165041, and 20 μmol/L of Wy14643. Treatment with PPAR agonists alone (in the absence of FH535) does not show a significant effect on β-catenin/Tcf activity relative to no treatment (data not shown).

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

they are activators of both pathways (5, 31, 32). PPARs recruitment of these factors and the corepressors NCoR and SMRT was studied using a mammalian two-hybrid assay, in which activation of a gal4-dependent reporter is regulated by the interaction between VP16-PPARs with the gal4-transcription factor chimera. FH535 inhibits GRIP1 but not the corepressors recruitment to PPARδ (Fig. 5A). A similar pattern of repressing coactivator recruitment, whereas allowing corepressor binding, was observed for GW9662 (26). However, the recruitment of β-catenin to PPARγ is inhibited by FH535 but not by GW9662 (Fig. 5B). In this regard, GW9662 behaves like the vitamin D receptor antagonist, ZK159222, that is unable to prevent the recruitment of β-catenin to vitamin D receptors, whereas maintaining the ability to inhibit the recruitment of other coactivators (33).

Because PPAR ligand regulates the transcription of only a subset of genes, we looked for genes targeted by FH535. We focused on genes most important for Wnt/β-catenin signaling. β-Catenin levels were unaffected by FH535 (data not shown), whereas TCF4 transcription was suppressed in FH535-treated HCT116 cells (Fig. 5C).

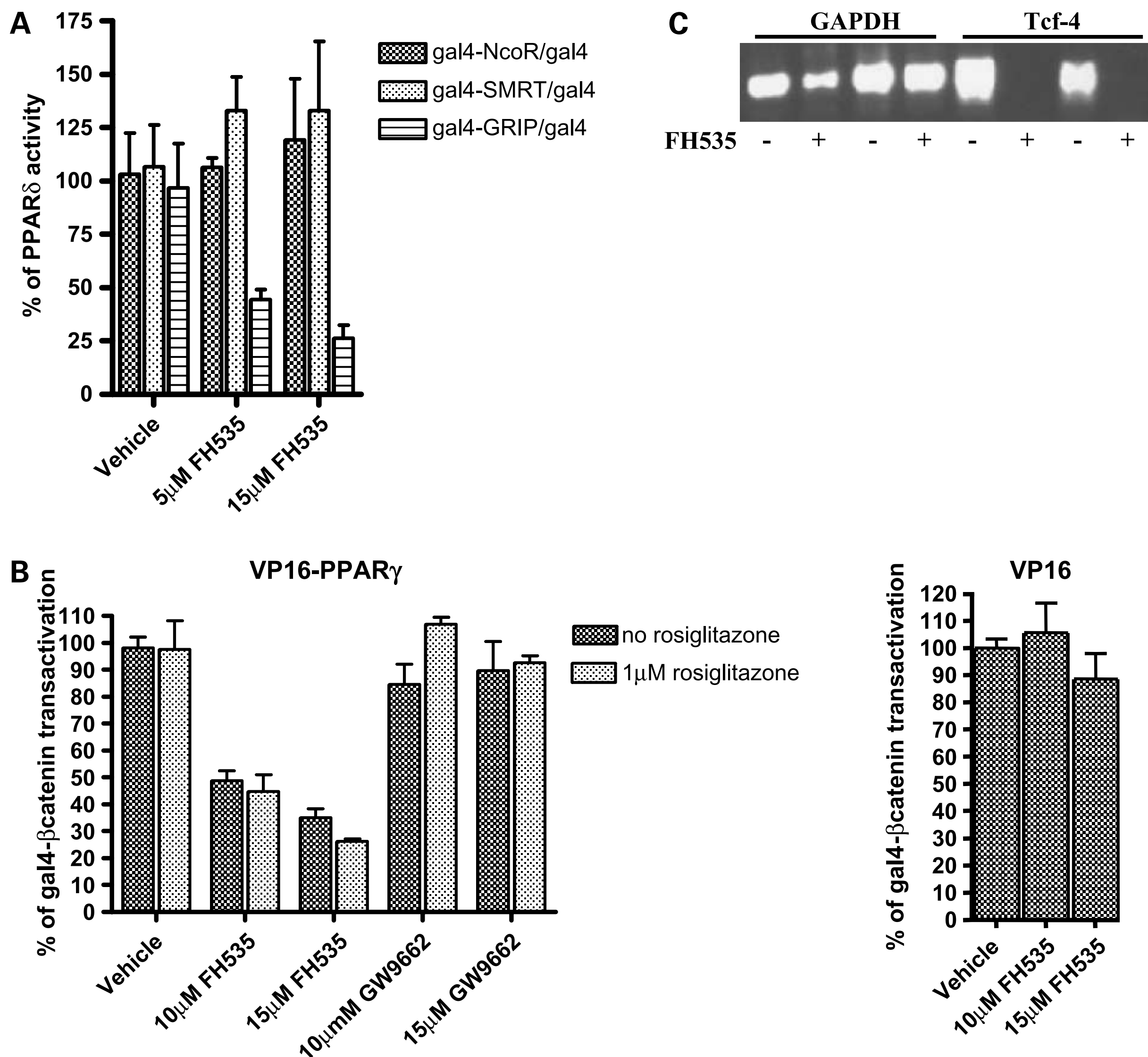

Figure 5. FH535 inhibits recruitment of the coactivators GRIP1 and β-catenin to PPARδ and PPARγ. **A,** FH535 inhibits PPARδ interaction with GRIP1 but not with NCoR or SMRT. HCT116 cells were transfected with a gal4-dependent luciferase reporter, VP16-PPARδ, and gal4-factor or gal4. The effects of FH535 on gal4-factor and gal4 were measured and the ratio of gal4-factor/gal4 was calculated. **B,** FH535 but not GW9662 inhibits the physical interaction between PPARγ and β-catenin. HCT116 cells were transfected with gal4-dependent luciferase reporter, gal4/β-catenin and either VP16-PPARγ or VP16. The effects of PPAR antagonists on gal4/β-catenin transactivation were measured in the presence or absence of 1 μmol/L of rosiglitazone. **C,** FH535 inhibits tcf4 transcription. The expression levels of GAPDH and Tcf4 in HCT116 cells treated with 15 μmol/L of FH535 or vehicle (DMSO) were determined by reverse transcription-PCR. Data from two independent compound treatments is shown. GAPDH served as an internal control.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

Table 1. LC_{50} values of FH535 in different cell lines grown in 10% FBS

Cell line	Cell type	Wnt/β-catenin signaling status*	LC_{50} (μmol/L)
HCT116	Colon adenocarcinoma	Elevated, mutant β-catenin	>30
SW48	Colon adenocarcinoma	Elevated, mutant β-catenin	>30
RKO	Colon carcinoma	Active, mutant CDX2	16
LoVo	Colon carcinoma	Elevated, mutant APC	12
COLO205	Colon carcinoma	Elevated, mutant β-catenin and APC	12
IEC6	Immortal small intestine	Inactive	>30
A427	Squamous lung carcinoma	Elevated, mutant β-catenin	10
HCC15	Lung adenocarcinoma	Elevated, mutant β-catenin	3.5
NCI-H1703	Squamous lung carcinoma	Elevated, high Dv13	5.5
A549	Large cell lung carcinoma	Active, high Wnt2	18
HepG2	Hepatocellular carcinoma	Elevated, mutant β-catenin	6.5
Hep3b	Hepatocellular carcinoma	Elevated, high nuclear β-catenin	5
Huh7	Hepatocellular carcinoma	Elevated, high nuclear β-catenin	15
Fibroblasts	Primary foreskin	Inactive	>30

NOTE: FH535 is selectively toxic to some colon, lung, and liver carcinomas expressing high or active Wnt/β-catenin pathways, but not to cells in which the Wnt/β-catenin signaling is not active.
*The levels of Wnt/β-catenin signaling (inactive, active, and elevated) in the different cell lines were determined using an optimized Tcf-binding reporter. The relevant known status of proteins responsible for Wnt/β-catenin signaling activation in the different cell lines is noted and compiled from published literature.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

FH535 Is Selectively Toxic to Some Carcinomas Expressing the Wnt/β-Catenin Pathway

Inhibition of Wnt/β-catenin signaling in some cancer cell lines that overexpress this pathway was shown to be toxic. For example, deletion of the activated mutant β-catenin in SW48 colon carcinoma cells is lethal but causes only a weak growth inhibition of HCT116 cells (34, 35). Because FH535 is an effective β-catenin/Tcf inhibitor (Fig. 1C), we tested its toxicity in 12 carcinoma cell lines expressing this signaling pathway (Table 1). The LC_{50} of most carcinomas tested against FH535 was 5 to 15 μmol/L. At this range, β-catenin/Tcf–dependent transactivation is inhibited by >50% (Figs. 1C and 4A). Only the two colon carcinomas, SW48 and HCT116, were resistant to the compound's toxicity at concentrations of up to 30 μmol/L. This suggests that FH535 inhibition of β-catenin/Tcf–dependent transactivation does not extend to the other oncogenic functions of β-catenin essential for the survival of SW48 cells. Interestingly, FH535 is toxic to A549 and RKO cell lines that express low levels of β-catenin/Tcf–dependent activity but respond to treatment with Wnt ligands. Cells that do not express the Wnt/β-catenin pathway, the primary fibroblasts and the immortal intestine cell line IEC6, were unaffected by FH535 at concentrations of up to 30 μmol/L.

Discussion

Several groups studying the therapeutic potential of PPARγ and PPARδ ligands to suppress Wnt/β-catenin pathway both in cell culture and in *in vivo* conditions reported conflicting results (reviewed in ref. 8). This suggests that only a specific type of PPAR modulation in a defined cellular environment could lead to the inhibition of Wnt/β-catenin pathway. Comparison of FH535 activities with other PPAR ligands provides an insight to the requirement for Wnt/β-catenin pathway inhibition.

FH535 and GW9662 share similar structure and both are antagonistic to PPARγ, but FH535 is unique in its ability to inhibit the Wnt/β-catenin pathway (Fig. 3C). One explanation for this difference is the target specificity of the compounds. FH535 antagonizes both PPARγ and PPARδ whereas GW9662 is specific for PPARγ. GW9662 is unable to reduce Tcf/β-catenin transactivation in cells expressing or lacking the *PPARδ* gene (Fig. 3C). In contrast, FH535 maintains partial antagonistic activity even in PPARδ-deficient cells (Fig. 3C). Thus, the inability of GW9662 to antagonize PPARδ cannot be the only reason for the difference between the two compounds, and suggests that they might also differ in the ability to inhibit the Wnt/β-catenin pathway via PPARγ.

GW9662 requires Cys^{285} to covalently bind PPARγ (26), whereas FH535's antagonistic activity does not depend on this cysteine residue (Fig. 3A). Similarly, the PPARγ agonist 15ΔPGJ2, but not rosiglitazone, use Cys^{285} for binding and transactivation (28). The nature of the interactions between the PPARγ ligands and specific residues in the PPARγ-binding domain leads to selectivity in coactivator recruitment. For example, recruitment of GRIP1 and SRC1 coactivators by 15ΔPGJ2 is superior compared with troglitazone, an analogue of rosiglitazone (31). Thus, lysophosphatidic acid, in contrast to the other PPAR agonists we tested (Fig. 3B), may direct the recruitment of specific coactivators that contribute to Wnt/β-catenin signaling regulation and are the targets of FH535 inhibition. Coactivator recruitment is believed to be a crucial step in PPAR-targeted gene activation because it is important for chromatin remodeling and for interaction with the basic transcription machinery (36). This suggests that the differences between FH535 and GW9662, with regard to binding PPARγ and β-catenin recruitment, may form the basis for the compounds' different abilities to regulate β-catenin/Tcf–dependent genes.

The ability of PPARγ to bind β-catenin and regulate β-catenin/Tcf activity is shared with other nuclear receptors like the orphan nuclear receptor LRH1 and the androgen receptor. LRH1 was suggested to serve as a direct coactivator for Tcf/β-catenin transactivation from the cyclin D1 promoter (37). Androgen receptor can sequester β-catenin from Tcf4 or promote β-catenin/Tcf4 interaction depending on the biological system analyzed (38, 39). The direct binding of androgen receptor to β-catenin and Tcf4 is thought to mediate the regulation of Wnt signaling. Like the androgen receptor, PPARγ interacts with β-catenin and is found in a complex with Tcf4 (5, 40). Thus, it is conceivable that FH535 inhibits a complex containing PPARγ β-catenin and Tcf/Lef proteins, depriving the complex bound to Tcf/Lef DNA sites from trans-activating. FH535 may achieve this by preventing the recruitment of GRIP1 to this complex because GRIP1 transactivates both PPARγ and Tcf/Lef proteins (31, 32). Alternatively, FH535 could suppress the transcription of selected PPARγ and PPARδ-targeted genes necessary for Wnt/β-catenin pathway activation. Tcf4 transcription is sensitive to FH535 inhibition and thus it is a good candidate for the transcriptional regulation of PPAR (Fig. 5C). The proposed mechanisms for FH535 activity are not mutually exclusive because the LRH1/β-catenin complex coactivates Tcf4 at the cyclin D1 promoter and also regulates cyclin E1 transcription by direct binding to the LRH1-RE site in the cyclin E1 promoter (37).

FH535 shows some functional similarity to R-etodolac, a stereoisomer of the NSAID etodolac. Unlike FH535, R-etodolac is a weak activator of the PPARγ-dependent reporter (16). However, in the presence of a strong PPARγ agonist, R-etodolac blocks the recruitment of the coactivator PBP by PPARγ. This antagonistic function of R-etodolac was suggested to explain its inhibition of the Wnt/β-catenin pathway (40). Diclofenac and indomethacin are also NSAIDs capable of both Wnt/β-catenin pathway inhibition and antagonizing strong activation of PPARγ (41, 42). All three compounds have been shown to bind and activate PPARγ. Thus, R-etodolac, diclofenac, and indomethacin are PPARγ ligands and may inhibit the Wnt/β-catenin pathway via their function as antagonists of PPARγ. Natural PPARγ agonists in the cancer cell environment could provide the necessary switch from a weakly agonistic to antagonistic function of these three compounds. The same natural PPARγ agonists can be expected to have the opposite effect on a true PPARγ antagonist's ability to suppress the Wnt/β-catenin pathway. This was observed for FH535 with the addition of either serum or lysophosphatidic acid (Fig. 4A and B).

FH535 inhibits Wnt/β-catenin signaling in cell lines that are resistant or sensitive to the toxic effect of FH535 (Fig. 1C; Table 1). FH535's effect on cell viability is complex and depends on several factors. First, inhibition of Wnt/β-catenin signaling is not toxic to all cell lines with elevated Wnt signaling (34, 35). Additionally, PPAR antagonists have general toxicity to some cells. This toxicity is independent of the ability to inhibit the Wnt/β-catenin signaling pathway because GW9662 and T0070907 are toxic to some cancer cell lines (43). Also, the effect of FH535 depends on the production and cellular concentration of PPAR agonists that antagonize its activity (Fig. 4B).

In this article, we show that FH535 is a tool to study the cross-interaction between the Wnt/β-catenin and the PPAR signaling pathways. FH535 is a more potent Wnt/β-catenin inhibitor than NO-aspirin (Fig. 1C), a leading experimental chemopreventive compound against colon cancer that inhibits intestinal tumors in APC^{min} mice (17). We expect that therapeutic concentration of FH535 can be achieved in mice because T0070907 was shown to reduce the metastasis of injected cancerous cells in mice (44) and GW9662 suppressed mice obesity induced by a high-fat diet (45).

Acknowledgments

We thank Sondra Goehle and Kevin Schutz for technical assistance.

References

1. Clevers H. Wnt/β-catenin signaling in development and disease. Cell 2006;127:469–80.

2. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and β-catenin signalling: diseases and therapies. Nat Rev Genet 2004;5:691–701.

3. Fodde R, Brabletz T. Wnt/β-catenin signaling in cancer stemness and malignant behavior. Curr Opin Cell Biol 2007;19:150–8.

4. Fuchs SY, Ougolkov AV, Spiegelman VS, Minamoto T. Oncogenic β-catenin signaling networks in colorectal cancer. Cell Cycle 2005;4:1522–39.

5. Jansson EA, Are A, Greicius G, et al. The Wnt/β-catenin signaling pathway targets PPARγ activity in colon cancer cells. Proc Natl Acad Sci U S A 2005;102:1460–5.

6. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell 1999;99:335–45.

7. Ross SE, Hemati N, Longo KA, et al. Inhibition of adipogenesis by Wnt signaling. Science 2000;289:950–3.

8. Muller R. Crosstalk of oncogenic and prostanoid signaling pathways. J Cancer Res Clin Oncol 2004;130:429–44.

9. Peraza MA, Burdick AD, Marin HE, Gonzalez FJ, Peters JM. The toxicology of ligands for peroxisome proliferator-activated receptors (PPAR). Toxicol Sci 2006;90:269–95.

10. Lefebvre AM, Chen I, Desreumaux P, et al. Activation of the peroxisome proliferator-activated receptor γ promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. Nat Med 1998;4:1053–7.

11. Saez E, Tontonoz P, Nelson MC, et al. Activators of the nuclear receptor PPARγ enhance colon polyp formation. Nat Med 1998;4:1058–61.

12. Niho N, Takahashi M, Shoji Y, et al. Dose-dependent suppression of hyperlipidemia and intestinal polyp formation in Min mice by pioglitazone, a PPARγ ligand. Cancer Sci 2003;94:960–4.

13. Gupta RA, Wang D, Katkuri S, Wang H, Dey SK, DuBois RN. Activation of nuclear hormone receptor peroxisome proliferator-activated receptor-δ accelerates intestinal adenoma growth. Nat Med 2004;10:245–7.

14. Marin HE, Peraza MA, Billin AN, et al. Ligand activation of peroxisome proliferator-activated receptor β inhibits colon carcinogenesis. Cancer Res 2006;66:4394–401.

15. Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J Biol Chem 1997;272:3406–10.

16. Hedvat M, Jain A, Carson DA, et al. Inhibition of HER-kinase activation prevents ERK-mediated degradation of PPARγ. Cancer Cell 2004;5:565–74.

17. Ouyang N, Williams JL, Rigas B. NO-donating aspirin isomers downregulate peroxisome proliferator-activated receptor (PPAR)δ expression in APC(min/+) mice proportionally to their tumor inhibitory effect: implications for the role of PPARδ in carcinogenesis. Carcinogenesis 2006;27:232–9.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024

18. Korinek V, Barker N, Morin PJ, et al. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC−/− colon carcinoma. Science 1997;275:1784 – 7.

19. Dufresne M, Jane D, Theriault A, Adeli K. Expression of cathepsin B and aryl hydrocarbon hydroxylase activities, and of apolipoprotein B in human hepatoma cells maintained long-term in a serum-free medium. In Vitro Cell Dev Biol Anim 1993;29A:873 – 8.

20. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-δ 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR γ. Cell 1995;83:803 – 12.

21. Slagsvold T, Kraus I, Fronsdal K, Saatcioglu F. DNA binding-independent transcriptional activation by the androgen receptor through triggering of coactivators. J Biol Chem 2001;276:31030 – 6.

22. Sadowski I. Ptashne MA vector for expressing GAL4(1 – 147) fusions in mammalian cells. Nucleic Acids Res 1989;17:7539.

23. Park BH, Vogelstein B, Kinzler KW. Genetic disruption of PPARδ decreases the tumorigenicity of human colon cancer cells. Proc Natl Acad Sci U S A 2001;98:2598 – 603.

24. Lamb JR, Goehle S, Ludlow C, Simon JA. Thymidine incorporation is highly predictive of colony formation and can be used for high-throughput screening. Biotechniques 2001;30:1118 – 20.

25. Nath N, Kashfi K, Chen J, Rigas B. Nitric oxide-donating aspirin inhibits β-catenin/T cell factor (TCF) signaling in SW480 colon cancer cells by disrupting the nuclear β-catenin-TCF association. Proc Natl Acad Sci U S A 2003;100:12584 – 9.

26. Leesnitzer LM, Parks DJ, Bledsoe RK, et al. Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662. Biochemistry 2002;41: 6640 – 50.

27. Lee G, Elwood F, McNally J, et al. T0070907, a selective ligand for peroxisome proliferator-activated receptor γ, functions as an antagonist of biochemical and cellular activities. J Biol Chem 2002;277:19649 – 57.

28. Shiraki T, Kamiya N, Shiki S, Kodama TS, Kakizuka A, Jingami H. α,β-Unsaturated ketone is a core moiety of natural ligands for covalent binding to peroxisome proliferator-activated receptor γ. J Biol Chem 2005; 280:14145 – 53.

29. Tsukahara T, Tsukahara R, Yasuda S, et al. Different residues mediate recognition of 1-O-oleyllysophosphatidic acid and rosiglitazone in the ligand binding domain of peroxisome proliferator-activated receptor γ. J Biol Chem 2006;281:3398 – 407.

30. McIntyre TM, Pontsler AV, Silva AR, et al. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARγ agonist. Proc Natl Acad Sci U S A 2003;100:131 – 6.

31. Kodera Y, Takeyama K, Murayama A, Suzawa M, Masuhiro Y, Kato S. Ligand type-specific interactions of peroxisome proliferator-activated receptor γ with transcriptional coactivators. J Biol Chem 2000;275: 33201 – 4.

32. Li H, Kim JH, Koh SS, Stallcup MR. Synergistic effects of coactivators GRIP1 and β-catenin on gene activation: cross-talk between androgen receptor and Wnt signaling pathways. J Biol Chem 2004;279:4212 – 20.

33. Shah S, Islam MN, Dakshanamurthy S, et al. The molecular basis of vitamin D receptor and β-catenin crossregulation. Mol Cell 2006;21: 799 – 809.

34. Kim JS, Crooks H, Foxworth A, Waldman T. Proof-of-principle: oncogenic β-catenin is a valid molecular target for the development of pharmacological inhibitors. Mol Cancer Ther 2002;1:1355 – 9.

35. Chan TA, Wang Z, Dang LH, Vogelstein B, Kinzler KW. Targeted inactivation of CTNNB1 reveals unexpected effects of β-catenin mutation. Proc Natl Acad Sci U S A 2002;99:8265 – 70.

36. Balint BL, Nagy L. Selective modulators of PPAR activity as new therapeutic tools in metabolic diseases. Endocr Metab Immune Disord Drug Targets 2006;6:33 – 43.

37. Botrugno OA, Fayard E, Annicotte JS, et al. Synergy between LRH-1 and β-catenin induces G1 cyclin-mediated cell proliferation. Mol Cell 2004; 15:499 – 509.

38. Song LN, Herrell R, Byers S, Shah S, Wilson EM, Gelmann EP. β-Catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription. Mol Cell Biol 2003;23:1674 – 87.

39. Singh R, Artaza JN, Taylor WE, et al. Testosterone inhibits adipogenic differentiation in 3T3-1 cells: nuclear translocation of androgen receptor complex with β-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors. Endocrinology 2006;147:141 – 54.

40. Lu D, Cottam HB, Corr M, Carson DA. Repression of β-catenin function in malignant cells by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 2005;102:18567 – 71.

41. Bishop-Bailey D, Warner TD. PPARγ ligands induce prostaglandin production in vascular smooth muscle cells: indomethacin acts as a peroxisome proliferator-activated receptor-γ antagonist. FASEB J 2003; 17:1925 – 7.

42. Adamson DJ, Frew D, Tatoud R, Wolf CR, Palmer CN. Diclofenac antagonizes peroxisome proliferator-activated receptor-γ signaling. Mol Pharmacol 2002;61:7 – 12.

43. Burton JD, Castillo ME, Goldenberg DM, Blumenthal RD. Peroxisome proliferator-activated receptor-γ antagonists exhibit potent antiproliferative effects versus many hematopoietic and epithelial cancer cell lines. Anticancer Drugs 2007;18:525 – 34.

44. Schaefer KL, Takahashi H, Morales VM, et al. PPARγ inhibitors reduce tubulin protein levels by a PPARγ, PPARδ and proteasome-independent mechanism, resulting in cell cycle arrest, apoptosis and reduced metastasis of colorectal carcinoma cells. Int J Cancer 2007; 120:702 – 13.

45. Nakano R, Kurosaki E, Yoshida S, et al. Antagonism of peroxisome proliferator-activated receptor γ prevents high-fat diet-induced obesity *in vivo*. Biochem Pharmacol 2006;72:42 – 52.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/3/521/1879390/521.pdf by guest on 13 October 2024