Activation of peroxisome proliferator-activated receptor γ does not explain the anti-proliferative activity of the non-steroidal anti-inflammatory drug indomethacin on human colorectal cancer cells

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JPET Fast Forward. Published on February 11, 2003 as DOI: 10.1124/jpet.103.048769
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Running title: Indomethacin and PPARγ in human colorectal cancer cells

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Number of text pages: 26

Number of tables: 0

Number of figures: 4

Number of references: 40

Number of words:

Abstract - 188

Introduction - 496

Discussion - 943

Abbreviations: ANOVA, analysis of variance; NSAID, non-steroidal anti-inflammatory drug; PPAR, peroxisome proliferator-activated receptor

Section assignment: Cellular and Molecular

JPET Fast Forward. Published on February 11, 2003 as DOI: 10.1124/jpet.103.048769
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Abstract

The mechanism of the anti-colorectal cancer activity of the non-steroidal anti-inflammatory drug indomethacin is poorly understood. Indomethacin inhibits both cyclooxygenase (COX) isoforms, but may also act via COX-independent targets. Indomethacin can bind and activate the transcription factor peroxisome proliferator-activated receptor (PPAR) γ. Moreover, natural and synthetic PPARγ ligands can induce growth arrest and apoptosis of human colorectal cancer cells *in vitro*. Therefore, we tested the hypothesis that the anti-proliferative activity of indomethacin on human colorectal cancer cells *in vitro* is explained by a PPARγ-dependent mechanism of action. Human colorectal cancer cell lines SW480 and HCT116 both expressed functional PPARγ. Indomethacin directly activated PPARγ in both cell lines (HCT116 > SW480). A dominant-negative PPARγ strategy was used to demonstrate that endogenous PPARγ represses proliferation of HCT116 cells (compatible with tumour suppressor activity) but that the presence of functional PPARγ is not necessary for the anti-proliferative activity (or reduction in cyclin D1 protein) associated with indomethacin *in vitro*. In summary, indomethacin (> 100 μM) directly activates PPARγ in human colorectal cancer cells. However, PPARγ activation does not underlie the anti-neoplastic activity of indomethacin on human colorectal cancer cells *in vitro*.

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A substantial body of evidence exists that non-steroidal anti-inflammatory drugs (NSAIDs) have anti-colorectal cancer (CRC) activity (Garcia Rodriguez and Huerta-Alvarez, 2001; Shiff and Rigas, 1997). However, the mechanism(s) of the anti-neoplastic activity of this class of drugs remains unclear. It is well recognised that the majority of NSAIDs inhibit one or both of the cyclooxygenase (COX) enzymes, COX-1 and COX-2 (Shiff and Rigas, 1999). As a role for the COX isoforms (particularly COX-2) has been implicated in the early stages of intestinal tumorigenesis and at later stages of colorectal carcinogenesis (Gupta and DuBois, 2001; Chulada et al., 2000), COX inhibition has generally been understood to underlie the anti-CRC activity of NSAIDs (Shiff and Rigas, 1999; Gupta and DuBois, 2001). However, several COX-independent mechanisms of action of NSAIDs have also been described in cultured CRC cells *in vitro* (Tegeder et al., 2001) and some NSAIDs, which lack COX-inhibitory activity, retain potent preventative properties in rodent colon carcinogenesis models *in vivo* (Shiff and Rigas, 1997). At present, the relative contributions of COX inhibition and COX-independent mechanisms, to the overall anti-CRC activity of individual NSAIDs, remain unclear (Marx, 2001).

The NSAID indomethacin has potent anti-CRC activity *in vitro* and *in vivo* (Tanaka et al., 1989; Hixson et al., 1994; Hirota et al., 1996; Smith et al., 2000; Chiu et al., 2000; Garcia Rodriguez and Huerta-Alvarez, 2001; Turchanowa et al., 2001; Brown et al., 2001). We, and others, have previously reported that indomethacin induces G1 growth arrest and apoptosis of several human CRC cell lines in a concentration-dependent manner *in vitro* (Hixson et al., 1994; Smith et al., 2000; Turchanowa et al., 2001). The anti-proliferative activity of indomethacin against human CRC cells does not require COX-2 inhibition (Smith et al., 2000) and indomethacin has been demonstrated to retain growth inhibitory and pro-apoptotic

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effects on transformed murine embryonic fibroblasts which lack either COX isoform (Zhang et al., 1999). Therefore, it is likely that COX-independent mechanisms contribute to the anti-CRC activity of indomethacin, at least *in vitro*.

A candidate target for COX-independent activity of indomethacin is the transcription factor peroxisome proliferator-activated receptor (PPAR) γ (Gupta and DuBois, 2002). Activation of PPARγ by synthetic ligands eg thiaziolidinediones (TZDs) or putative endogenous ligands eg. 15-d-prostaglandin (PG) J_2 lead to growth arrest and differentiation of several cell types, including human CRC cell lines (Brockman et al., 1998; Shimada et al., 2002; Gupta and DuBois, 2002). Importantly, indomethacin (and, to a lesser extent, other NSAIDs eg. ibuprofen) can directly bind and activate PPARγ in monkey CV-1 cells (Lehmann et al., 1997) and human rheumatoid synovial cells (Yamazaki et al., 2002). Alternatively, COX inhibition by indomethacin could lead to decreased synthesis of cyclopentenone PGs such as 15-d-PGJ_2 and hence attenuation of PPARγ activity. The contribution, if any, of these two opposing effects of indomethacin on PPARγ in malignant colorectal epithelial cells is not known.

Therefore, we tested the hypothesis that the anti-proliferative activity of indomethacin against human CRC cells is explained by a PPARγ-dependent mechanism of action.

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Methods

Cell culture

The human sporadic CRC cell lines SW480 and HCT116 (European Collection of Animal Cell Cultures, Porton Down, UK) were cultured in RPMI 1640 medium containing Glutamax-I™, supplemented with 10% (v/v) foetal bovine serum, 1000 U/ml penicillin and 500 U/ml streptomycin (all Life Technologies, Paisley, UK), on tissue culture plastic, as described (Smith et al., 2000; Hawcroft et al., 2002).

Drugs, antibodies and DNA plasmids

Indomethacin (Sigma) was prepared as a 100 mM stock solution in dimethyl sulphoxide (DMSO; Sigma, Poole, UK). Troglitazone (a kind gift from Parke-Davis Pharmaceutical Research, Ann Arbor, MI) was prepared as a 10 mM stock solution in DMSO. In experiments testing the effects of these drugs, control cell cultures always contained an equivalent v/v dilution of DMSO to that in cultures which contained the highest concentration of drug.

Mouse monoclonal anti-human PPARγ antibody (E8) and mouse monoclonal anti-human cyclin D1 antibody (A-12) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti-human β-actin antibody (AC-15) and anti-FLAG (M2) antibody were obtained from Sigma. Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody was obtained from DAKO Ltd. (Ely, UK).

A pCMX vector containing a peroxisome proliferator response element (x3)-*luciferase* reporter gene (*PPRE3-tk-luciferase*) was a kind gift from R. Evans, The Salk Institute, San Diego, CA (Forman et al., 1995). Plasmid pcDNA3 containing

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FLAG epitope-tagged human *PPARγ1* with a L468A/E471A double mutation in the AF-2 domain (thus producing dominant-negative PPARγ activity) was a kind gift from K. Chatterjee, Cambridge, UK. (Gurnell et al., 2000). pRL-TK *Renilla luciferase*-reporter gene plasmid was obtained from Promega (Madison, WI).

Reverse transcription-polymerase chain reaction analysis of PPARγ mRNA expression

Total RNA was prepared from SW480 and HCT116 cells using RNeasy columns (Qiagen Ltd., Crawley, UK) as per manufacturer's instructions and then reverse-transcribed. Polymerase chain reaction (PCR) amplification for human PPARγ was performed as described (Brockman et al., 1998), giving an amplicon size of 234 bp. PCR products were subjected to electrophoresis on 2% agarose in the presence of 0.5 mg ml^{-1} ethidium bromide (Sigma).

Western blot analysis

Cell monolayers were lysed using 50 mM Tris-HCl pH 7.2, 0.137 M NaCl containing 1% (v/v) Brij 96 (all Sigma) as described (Smith et al., 2000; Hawcroft et al., 2002) or Passive Lysis Buffer (Promega). The total protein concentration of lysate supernatants was determined using the Bio-Rad DC protein assay (Bio-Rad, Hemel Hempstead, UK). NUPAGE® NOVEX 10% Bis-Tris 1mm gels in 1x NU-PAGE® 3-(N-Morpholino) propanesulphonic acid (MOPS) running buffer (all Invitrogen, Paisley UK) were used to resolve 20 μg total protein samples and a MagicMark™ Western molecular weight standard (Invitrogen). Proteins were transferred to Hybond P polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Amersham,

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UK) using a XCell II™ wet blot module with 1x NU-PAGE® transfer buffer (Invitrogen). Membranes were blocked with a 5% (w/v) solution of non-fat skimmed milk powder in PBS containing 0.02% (v/v) Tween 20 (PBS/T) for 1 hr at 20°C, prior to incubation with primary antibodies (anti-PPARγ, 1:200; anti-β-actin, 1:1000; or anti-cyclin D1, 1:1000 in PBS/T plus 5% (w/v) non-fat skimmed milk powder) for 1 hr at 20°C. Subsequently, three washes with PBS containing 0.05% (v/v) Tween 20 were followed by incubation with secondary antibody (1:5000 in PBS/T plus 5% non-fat skimmed milk powder) for 1 hr at 20°C. After three further washes with PBS containing 0.05% (v/v) Tween 20, enhanced chemiluminescence was detected as per manufacturer's instructions (Perbio Science, Tattenhall, UK).

Transient DNA transfection and dual luciferase reporter assays

GeneJuice™ transfection reagent (Novagen) was added to serum-free RPMI 1640 containing Glutamax-I™ and incubated for 5 min prior to addition of the appropriate DNA. The GeneJuice™-DNA mix was incubated for 30 min at 20°C before addition to 35 mm well cell cultures (30-50% confluent) in RPMI 1640 medium containing Glutamax I™, supplemented with 2.5% (v/v) foetal bovine serum, 250 U/ml penicillin and 125 U/ml streptomycin. Medium was removed and fresh medium (RPMI 1640 medium containing Glutamax-I™, supplemented with 10% (v/v) foetal bovine serum, 1000 U/ml penicillin and 500 U/ml streptomycin) containing drug or carrier control was added 24 hr later. After a further 24 hr, dual-luciferase reporter assays (Promega) were performed as described (Hawcroft et al., 2002). Experiments were performed in triplicate and all data are expressed as the mean (+ standard error of the mean [SEM]) *Firefly luciferase* activity relative to

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Renilla luciferase activity. Cell lysates were used for subsequent Western blot analysis.

The cell proliferation assay

Cells were plated at 5 x 10^4 cells/well in 24-well plates. At 72 hr, cells were transfected with *PPARγΔAF-2* (4 μg) or mock transfected as above. After 24 hr, indomethacin (600 μM) or carrier control was added and the cells were incubated for a further 24 hr. Adherent cells were then harvested using 0.25% (w/v) trypsin and 1 mM ethylenediaminetetra-acetic solution (Life Technologies). Cell number and viability was measured using a haemocytometer and exclusion of 0.4% trypan blue (Sigma) in PBS. All conditions were assayed in triplicate.

Statistical analysis

Student's independent sample t-test was used for pair-wise comparisons. One-way analysis of variance (ANOVA), with post hoc Bonferroni tests, was used for multiple comparisons. Statistical significance was assumed if the p value was less than 0.05. All analyses were performed using SPSS (v11) computer software.

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Results

HCT116 and SW480 human CRC cells express functional PPARγ

Firstly, we determined whether HCT116 and SW480 human CRC cells expressed functional PPARγ. These two cell lines were chosen for these PPARγ studies as we had previously demonstrated that they were sensitive to the growth inhibitory and pro-apoptotic effects of indomethacin (100-600 μM; Smith et al., 2000). HCT116 and SW480 cells contain COX-1 but do not express COX-2 constitutively (Smith et al., 2000), which mirrors the phenotype of intestinal epithelial cells at the earliest stages of colorectal carcinogenesis of relevance to CRC chemoprevention (Chapple et al., 2002). HCT116 and SW480 cell lines both expressed PPARγ mRNA and protein (figure 1*a* and *b*). The HCT15 human CRC cell line also expresses PPARγ protein but it exists in a functionally inactive state in these cells (Brockman et al., 1998). Therefore, we confirmed the functional status of PPARγ in HCT116 and SW480 cells by testing that activation of PPARγ by the TZD troglitazone led to increased *PPRE-luciferase* reporter gene activity in HCT116 and SW480 cells. Troglitazone (10-50 μM) significantly increased *PPRE-luciferase* reporter gene activity in both cell lines, in a concentration-dependent manner (figure 1*c*). *PPRE-luciferase* gene *trans*-activation induced by troglitazone was significantly greater in HCT116 cells than SW480 cells (figure 1*c*).

Indomethacin activates PPARγ in HCT116 and SW480 human CRC cells

Indomethacin induces G1 arrest and apoptosis of human CRC cells, including HCT116 and SW480 cells in a concentration-dependent manner, at concentrations greater than 100 μM (Hixson et al., 1994; Smith et al., 2000; Turchanova et al., 2001). These effects are demonstrable from 24 hr onwards (Smith et al., 2000). In our

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previous studies, maximal effects of indomethacin on proliferation and apoptosis of human CRC cells were observed at 600 μM (Smith et al., 2000). At this concentration of indomethacin, we have also previously observed specific down-regulation of β-catenin protein levels (but not of its homologue γ-catenin) in SW480 and HCT116 cells (Hawcroft et al., 2002). Therefore, we initially tested the ability of this concentration of indomethacin to activate PPARγ in these human CRC cell lines. Indomethacin treatment (600 μM) significantly increased *PPRE-luciferase* activity in both cell lines (figure 2*a*). In a similar manner to troglitazone (figure 1*c*), the fold increase in PPARγ activation by indomethacin was greater in HCT116 cells than SW480 cells (figure 2*a*). Therefore, we used HCT116 cells as model human CRC cells in all subsequent experiments. Indomethacin induced PPARγ activation in a concentration-dependent manner (figure 2*b*) with maximal activation occurring at a concentration of 300 μM. Importantly, 10 μM indomethacin (a concentration below that required for significant, direct PPARγ activation [Lehmann et al., 1997; Jaradat et al., 2001; Yamazaki et al., 2002], but at which profound COX inhibition in human CRC cells occurs [Kokoska et al., 1999]) did not significantly alter basal PPARγ activity in HCT116 cells, with only a slight increase in *PPRE-luciferase* activity being apparent (figure 2*b*). This suggests that down-regulation of PPARγ activity, via inhibition of COX-1-derived PG PPARγ ligand synthesis, does not occur at lower concentrations of indomethacin in HCT116 cells.

The anti-proliferative activity of indomethacin is not dependent on PPARγ activation

We then used mutant dominant-negative human PPARγ (PPARγΔAF-2) in order to antagonize endogenous PPARγ activity and test the functional relationship

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between direct PPARγ activation and the anti-proliferative effects of indomethacin. Firstly, we confirmed that PPARγΔAF-2 had dominant-negative activity in HCT116 cells. PPARγΔAF-2 expression significantly decreased basal and TZD-induced *trans*-activation of the *PPRE-luciferase* reporter gene in HCT116 cells by approximately 80% (figure 3). We tested the anti-proliferative activity of 600 μM indomethacin on HCT116 cells, as this concentration of indomethacin induces maximal growth arrest and apoptosis of HCT116 cells at 24 hrs (the most relevant time point for the transiently transfected cells in the current experiments; Smith et al., 2000). Indomethacin treatment induced a 65% decrease in control HCT116 cell number at 24 hrs (figure 4). The presence of the transfection reagent alone did not alter HCT116 cell proliferation (figure 4). Expression of PPARγΔAF-2 in HCT116 cells was confirmed by Western blot analysis of the FLAG epitope (figure 4). Dominant-negative PPARγ expression was associated with an increase in HCT116 cell proliferation (figure 4), which approached statistical significance ($p = 0.11$). However, the anti-proliferative effect of indomethacin was not altered in the presence of dominant-negative PPARγ (figure 4). Indomethacin treatment has been associated with down-regulation of the cell cycle gene *cyclin D1* in human CRC cells (Hawcroft et al., 2002) and PPARγ activation has been demonstrated to repress *cyclin D1* expression in HeLa cells (Wang et al., 2001). Therefore, we also investigated whether decreased *cyclin D1* expression associated with indomethacin treatment was dependent on endogenous PPARγ activation. In keeping with the lack of effect of dominant-negative PPARγ on the anti-proliferative effects of indomethacin, PPARγΔAF-2 did not abrogate the decrease in cyclin D1 protein levels associated with indomethacin treatment (figure 4).

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Discussion

We have demonstrated that indomethacin can activate PPARγ in human CRC cells but that this pharmacological mode of action does not contribute to the anti-proliferative activity of indomethacin on human CRC cells *in vitro*.

Previous data showing direct binding and transcriptional activation of PPARγ by indomethacin has been obtained from experiments on cells (CV-1 and COS-1 cells transfected with human PPARγ, as well as human rheumatoid synoviocytes) with little relevance to CRC (Lehmann et al., 1997; Jaradat et al., 2001; Adamson et al., 2002; Yamazaki et al., 2002). This study has provided definitive evidence that indomethacin also has similar activity in human CRC cells *in vitro*, at similar (> 100 μM) concentrations to those previously reported to activate PPARγ (Lehmann et al., 1997; Jaradat et al., 2001; Adamson et al., 2002; Yamazaki et al., 2002). Importantly, negative regulation of PPARγ activity at lower concentrations of indomethacin (10 μM), compatible with significant COX inhibition in human CRC cells (Kokoska et al., 1999) and other cultured cell types (Kirtikara et al., 2001), but minimal direct PPARγ activation (Lehmann et al., 1997; Jaradat et al., 2001; Yamazaki et al., 2002), did not occur in HCT116 cells. The possibility that lower concentrations of indomethacin and other NSAIDs may decrease cyclopentenone PG (eg 15-d-PGJ_2) synthesis, and hence abrogate PPARγ activity, requires further investigation in other human CRC cell lines with different COX-1 and COX-2 expression profiles.

Both troglitazone and indomethacin produced more potent PPARγ activation in HCT116 cells than SW480 cells, despite similar levels of PPARγ mRNA and protein in these two human CRC cell lines. This suggests a human CRC cell line-specific difference in PPARγ function, which has previously been described in HCT15 human CRC cells (Brockman et al., 1998). Differential PPARγ activity may be related to the

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phosphorylation status of PPARγ (Adams et al., 1997) or heterozygous 'loss-of-function' mutation of *PPARγ* (Sarraf et al., 1999).

There was a biphasic response of the *PPRE-luciferase* reporter gene to indomethacin with maximal PPARγ activation at a concentration of 300 μM. A similar biphasic PPARγ activation pattern, with diminished *PPRE*-reporter gene activity at NSAID concentrations above 300 μM, has previously been described in transfected CV-1 cells treated with indomethacin or ibuprofen (Jaradat et al., 2001). At present, the explanation for this phenomenon is unclear.

It remains controversial what NSAID concentrations are achievable in colorectal mucosa and whether *in vitro* studies employing high NSAID concentrations are relevant to NSAID chemoprevention *in vivo* (Marx, 2001). Plasma indomethacin levels between 1 and 10 μM are obtained after acute dosing (200 mg) in humans (Hucker et al., 1966). However, indomethacin may undergo entero-hepatic circulation which could lead to intestine luminal drug levels significantly higher than plasma values (Hucker et al., 1966; Kwan et al., 1976). For example, the sulphone metabolite of the related NSAID sulindac can attain colorectal mucosal levels of 50-100 μM in humans (personal communication; R Pamukcu). Therefore, indomethacin concentrations capable of significant, direct PPARγ activation could be generated in human colonic mucosa.

We used a dominant-negative approach in order to test the relevance of PPARγ activation to the anti-neoplastic effects of indomethacin on human CRC cells *in vitro*. Mutant dominant-negative PPARγΔAF-2 has potent inhibitory activity in human CRC cells and is a potent tool for studying PPARγ-mediated gene *trans*-activation in a host of relevant human cell types. Although dominant-negative PPARγ did not affect the anti-proliferative activity of indomethacin, the basal proliferation rate of HCT116

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cells was increased in cells expressing dominant-negative PPARγΔAF-2. This implies that PPARγ signalling may down-regulate the proliferation rate of HCT116 cells and is in keeping with data that demonstrate that TZD-induced PPARγ activation leads to growth arrest and apoptosis of human CRC cells (Brockman et al., 1998; Shimada et al., 2002). These data are relevant to the continuing controversy about the role of PPARγ during colorectal carcinogenesis and its potential as a chemoprevention/chemotherapy target (Gupta and DuBois, 2002). Girnun and colleagues have recently described a complementary approach to our dominant-negative PPARγ strategy in which they tested the effect of *Pparγ* haplo-insufficiency (homozygous deletion of *Pparγ* is embryonic lethal) on colorectal carcinogenesis in mice (Girnun et al, 2002). $Ppar^{+/-}$ mice had an increased incidence of azoxymethane-induced colonic tumours compared with $Ppar^{+/+}$ littermates. Therefore, our data on human CRC cell proliferation are consistent with the tumour suppressor activity of Pparγ described in this *in vivo* model.

Several other effects of indomethacin on human CRC cells have previously been described (Tegeder et al., 2001). These include not only COX inhibition but also alterations in WNT signalling (Dihlmann et al., 2001; Hawcroft et al., 2002), BAX-mediated apoptosis (Zhang et al., 2000), increased p38 mitogen-activated protein kinase signalling (Kim et al., 2001), induction of NSAID-associated gene-1 expression (Kim et al., 2002; Baek et al., 2002), decreased ornithine decarboxylase activity (Turchanowa et al., 2001) and induction of nerve growth factor-induced gene B (Kang et al., 2000). In only some instances, the functional relevance of these findings to the anti-neoplastic activity of indomethacin *in vitro* and *in vivo* has been proven (Zhang et al., 2000; Kim et al., 2002). Our data strongly suggest that PPARγ activation is not necessary for the anti-proliferative action of indomethacin on human

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CRC cells *in vitro*. It will now be important to compare the chemopreventative activity of indomethacin in azoxymethane-treated *Ppar*$^{+/-}$ and *Ppar*$^{+/+}$ mice (Girnun et al., 2002) in order to confirm our findings using an *in vivo* model.

In summary, we have provided evidence that PPARγ activation does not underlie the anti-proliferative activity of indomethacin on human CRC cells *in vitro*. We have also confirmed previous data, based on PPARγ activation experiments, that PPARγ has tumour suppressor activity in human CRC cells, using a novel dominant-negative PPARγ strategy.

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Footnotes

This work was funded by Yorkshire Cancer Research and NHS Northern & Yorkshire R&D. The salary of Dr Gillian Hawcroft was obtained from Yorkshire Cancer Research and The West Riding Medical Research Trust. Dr Sarah Gardner is funded by NHS Northern & Yorkshire R&D. Dr Mark Hull holds a MRC (UK) Clinician Scientist Fellowship.

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JPET Fast Forward. Published on February 11, 2003 as DOI: 10.1124/jpet.103.048769
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Legends for figures

Figure 1

SW480 and HCT116 human CRC cells express functional PPARγ. (*a*) RT-PCR for PPARγ mRNA using SW480 and HCT116 cell total RNA. (*b*) Western blot analysis of PPARγ (48 kD) and β-actin (42 kD) protein expression in SW480 and HCT116 cell lysates. (*c*) SW480 and HCT116 cells contain functional PPARγ. A *PPRE3-tk-luciferase* reporter (1μg) was transiently co-transfected with a *Renilla-tk-luciferase* reporter (0.5 μg) into SW480 or HCT116 cells which were then treated with the PPARγ ligand troglitazone (0-50 μM) for 24 hr. Cells were lysed and dual-luciferase assays were performed. Data from triplicate experiments are expressed as the mean (+ SEM) of the ratio of PPRE reporter *Firefly luciferase* activity to control *Renilla luciferase* activity. * $p < 0.05$, ** $p < 0.01$ compared with control values (ANOVA).

Figure 2

Indomethacin activates PPARγ in SW480 and HCT116 human CRC cells. (*a*) *PPRE3-tk-luciferase* reporter (1μg) was transiently co-transfected with a *Renilla-tk-luciferase* reporter (0.5 μg) into SW480 or HCT116 cells. Cells were then treated with carrier control (□) or 600 μM indomethacin (■) for 24 hr. (*b*) *PPRE3-tk-luciferase* reporter (1μg) was transiently co-transfected with a *Renilla-tk-luciferase* reporter (0.5 μg) into HCT116 cells which were then treated with 30 μM troglitazone (trog) or indomethacin (10-600 μM) for 24 hr. In both *a*) and *b*), cells were lysed and dual-luciferase assays were performed 24 hr after drug treatment. Data from triplicate experiments are expressed as the mean (+ SEM) of the ratio of PPRE reporter *Firefly*

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luciferase activity to control *Renilla luciferase* activity. * $p < 0.05$, ** $p < 0.01$ compared with control values (*a*, Student's t-test; *b*, ANOVA)

Figure 3

Dominant-negative PPARγ inhibits endogenous PPARγ activity in HCT116 human CRC cells. A *PPRE3-tk-luciferase* reporter (1μg) and a *Renilla-tk-luciferase* reporter (0.5 μg) were transiently co-transfected in the absence (-) or presence (+) of *PPARγΔAF-2* (4 μg) into HCT116 cells. Cells were treated with carrier control (□) or 30 μM troglitazone (■) for 24 hr. Cells were then lysed and dual-luciferase assays performed. Data from triplicate experiments are expressed as the mean (+ SEM) of the ratio of PPRE reporter *Firefly luciferase* activity to control *Renilla luciferase* activity. * $p < 0.01$ compared with respective cells treated with carrier control or troglitazone, in the absence of *PPARγΔAF-2* (Student's t-test).

Figure 4

Indomethacin decreases HCT116 human CRC cell proliferation and cyclin D1 protein levels despite inhibition of endogenous PPARγ activation by dominant-negative PPARγ. HCT116 cells were transiently transfected with (4 μg) dominant-negative FLAG epitope-tagged *PPARγ(PPARγΔAF-2*) or mock-transfected and then treated with carrier control (□) or 600 μM indomethacin (■) for 24 hr. The viable cell number was counted in triplicate wells and the data are expressed as the mean (+ SEM) cell number. Western blot analysis was performed for cyclin D1 (34 kD), FLAG-PPARγΔAF-2 (54 kD) and β-actin (42 kD). * $p = 0.11$ compared with the number of carrier control-treated cells in the presence of transfection reagent (Student's t-test).

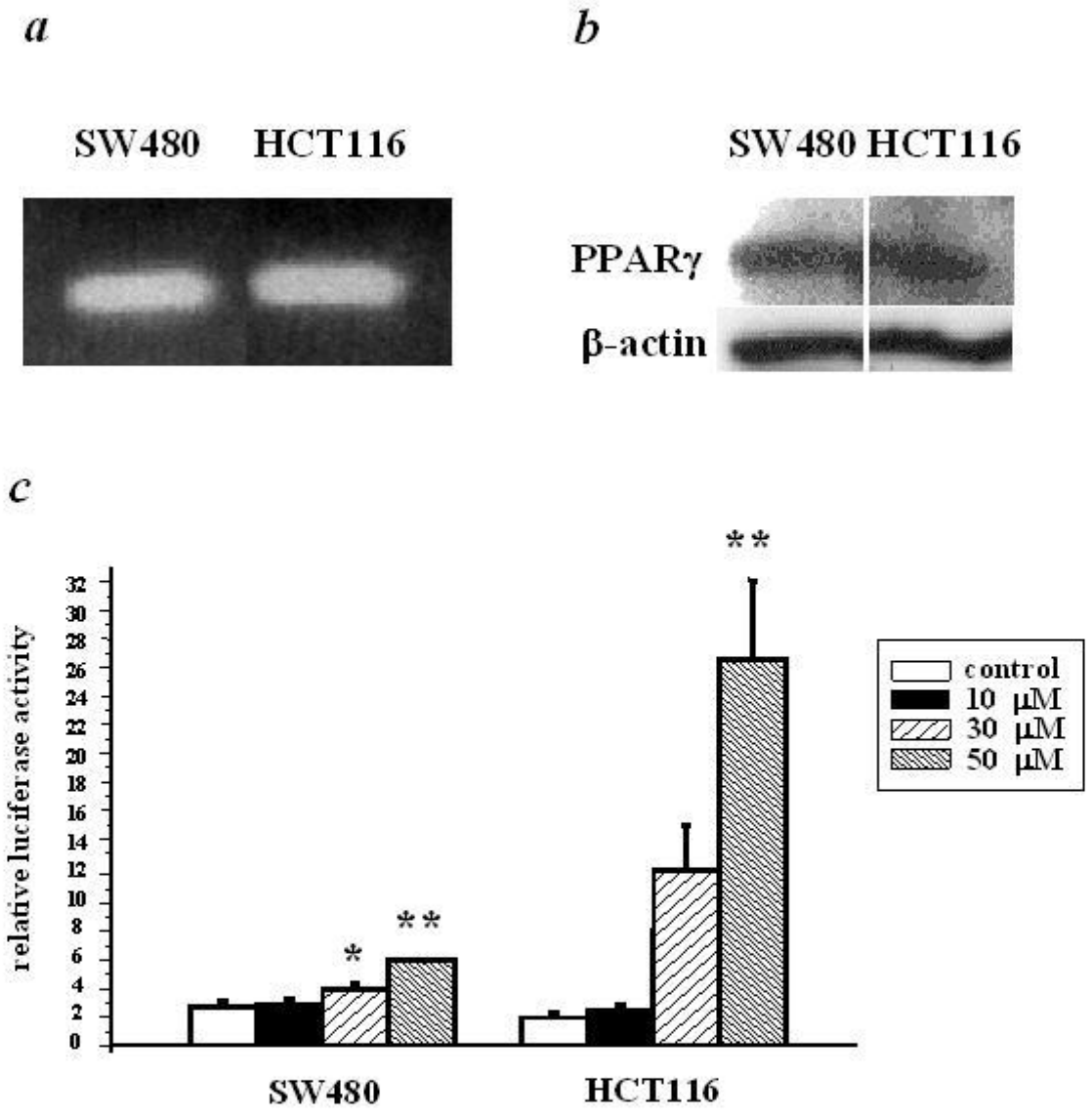
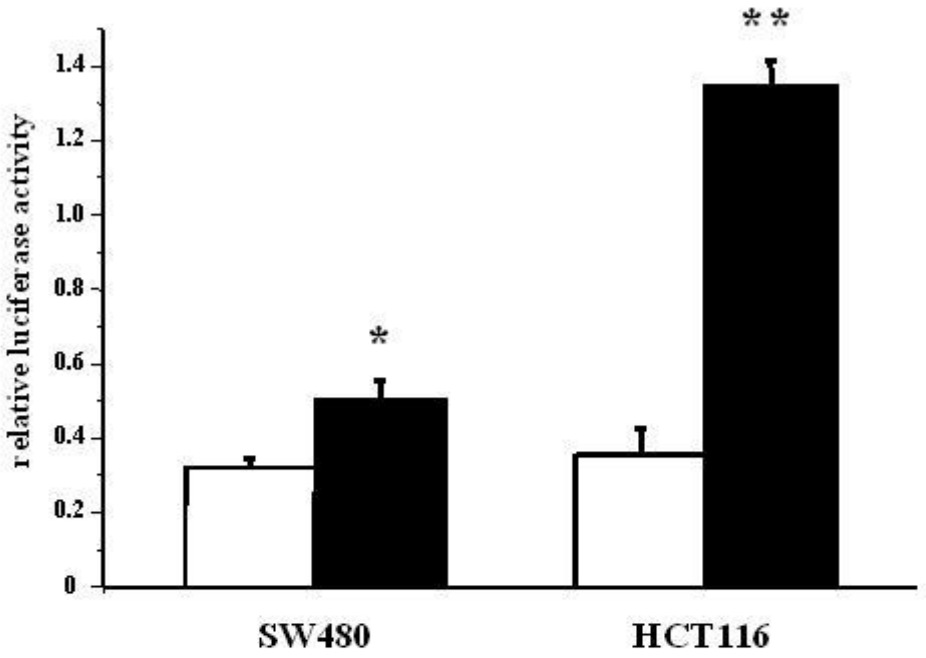

a
SW480
HCT116
b
SW480 HCT116
PPARγ
β-actin
c
relative luciferase activity
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
*
**
**
control
10 μM
30 μM
50 μM
SW480
HCT116

Figure 1

a


relative luciferase activity
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
**
SW480
HCT116

b

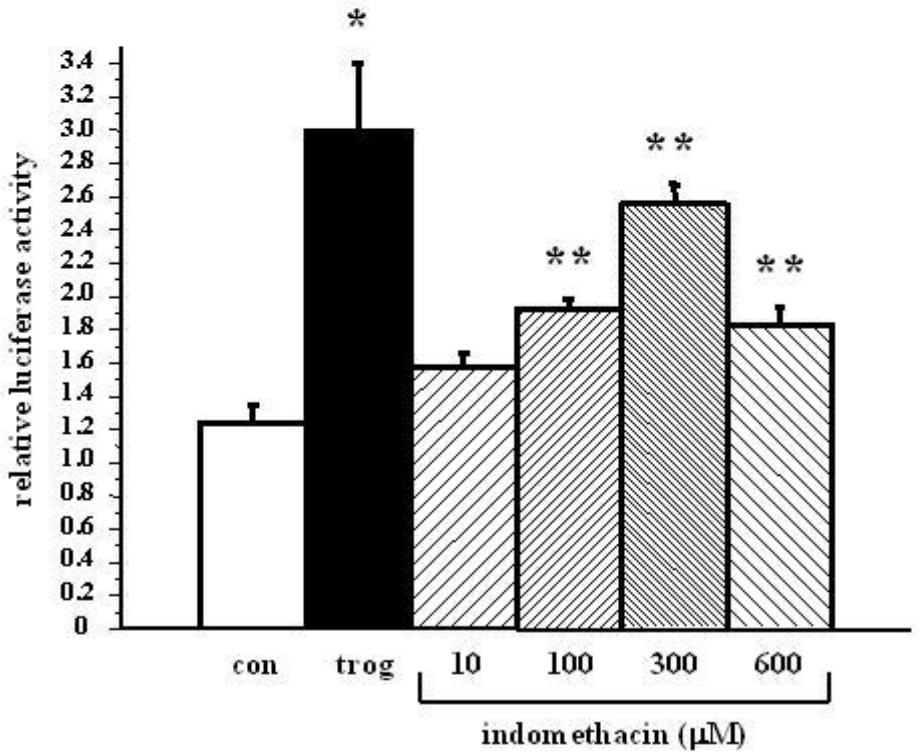

relative luciferase activity
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
*
**
**
**
con
trog
10
100
300
600
indomethacin (μM)

Figure 2

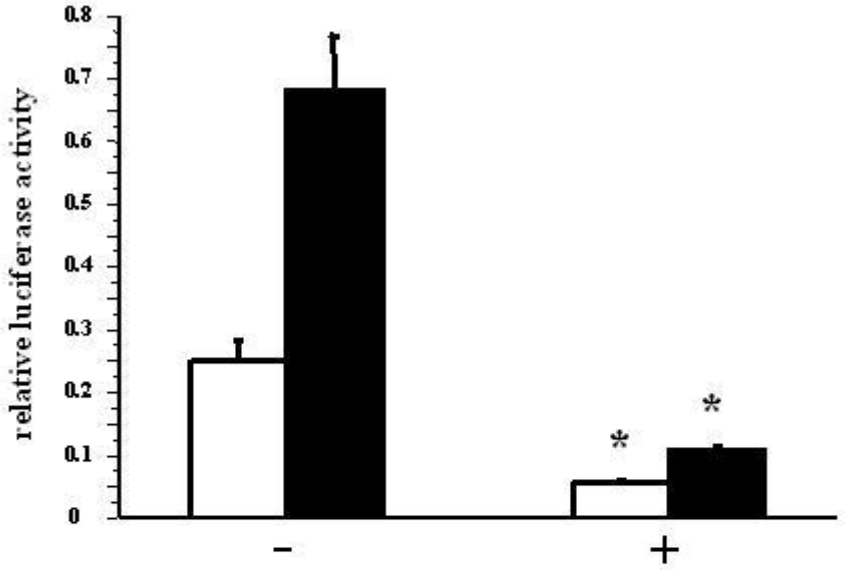


Figure 3

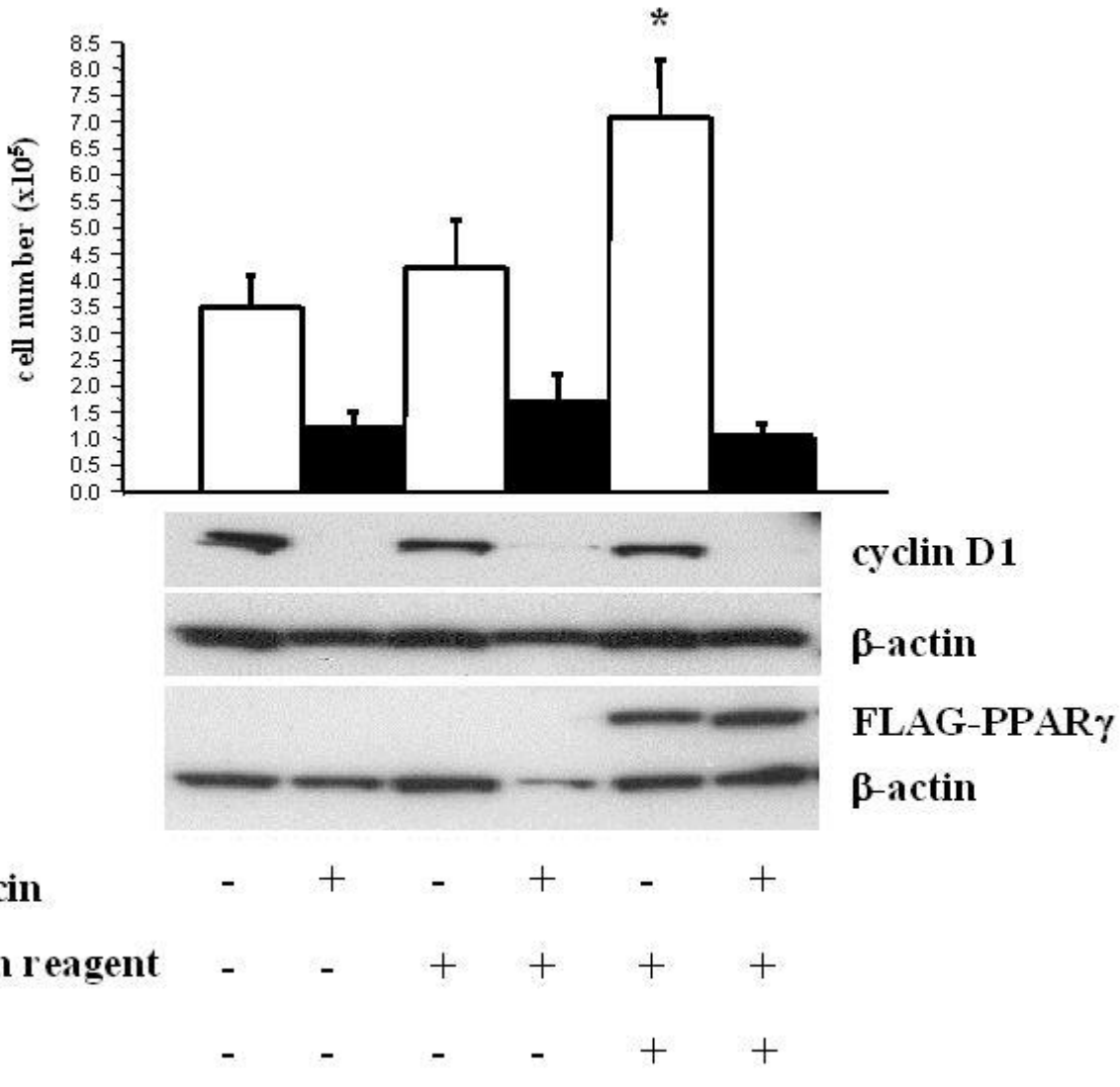

*
cell number (x10⁵)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cyclin D1
β-actin
FLAG-PPARγ
β-actin
indomethacin - + - + - +
transfection reagent - - + + + +
dnPPARγ - - - - + +

Figure 4