ACCELERATED COMMUNICATION

Diclofenac Antagonizes Peroxisome Proliferator-Activated Receptor-γ Signaling

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ABSTRACT

Although nonsteroidal anti-inflammatory drugs (NSAIDs) are used as cancer chemopreventative agents, their mechanism is unclear because NSAIDs have cyclooxygenase-independent actions. We investigated an alternative target for NSAIDs, peroxisome proliferator-activated receptor-γ (PPARγ), activation of which decreases cancer cell proliferation. NSAIDs have been shown to activate this receptor, but only at high concentrations. Here, we have examined binding of diclofenac to PPARγ using a *cis*-parinaric acid displacement assay and studied the effect of diclofenac effect on PPARγ *trans*-activation in a COS-1 cell reporter assay. Unexpectedly, diclofenac bound PPARγ at therapeutic concentrations (K_i = 700 nM) but induced only 2-fold activation of PPARγ at a concentration of 25 μM and antagonized PPARγ *trans*-activation by rosiglitazone. This antagonism was overcome with increasing rosiglitazone concen-

trations, indicating that diclofenac is a partial agonist. No effect of diclofenac was seen without exogenous receptor, confirming that it was working through a PPARγ-specific mechanism. This is the first description of an NSAID that can antagonize PPARγ. In addition, this is the first time that an NSAID has been shown to bind this receptor at clinically meaningful concentrations. The physiological relevance of these findings was tested using adipocyte differentiation and cancer cell proliferation assays. Diclofenac decreased PPARγ-mediated adipose cell differentiation by 60% and inhibited the action of rosiglitazone on the prostate cancer cell line, DU-145, allowing a 3-fold increase in proliferation. This work shows that standard doses of diclofenac may have pharmacodynamic interactions with rosiglitazone and this has therapeutic implications, both in the management of type 2 diabetes and during cancer treatment.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to modulate tumor cell growth in vitro for several decades (Lynch et al., 1978) and have shown promise clinically as chemopreventative agents against colon cancer (Turner and Berkel, 1993). NSAIDs are known to inhibit cyclooxygenase (COX) 1 and 2 and therefore block the inflammatory prostaglandin elements of the eicosanoid cascade. However, there is evidence that NSAIDs may act as chemoprotective agents through targets other than COX (Elder and Paraskeva, 1998). The nuclear receptor PPARγ is one such target that has been shown to have a role in inhibiting cancer cell growth (Mueller et al., 1998; Chang and Szabo, 2000), although little is known about the interaction between

NSAIDs and this nuclear receptor in the modulation of cancer cell growth.

PPARγ is a member of the steroid hormone receptor superfamily, which includes the thyroid hormone and estrogen receptors (Anonymous, 1999). Binding of a thiazolidinedione such as rosiglitazone to PPARγ allows it to form a heterodimer with the retinoid X receptor and activate downstream target genes. Such regulation controls the expression of genes involved in fatty acid metabolism such as acyl coenzyme A synthase, lipoprotein lipase, and leptin (Vamecq and Latruffe, 1999). PPARγ controls the differentiation of several cell types, such as adipocytes and macrophages, and also seems to modulate apoptosis (Gelman et al., 1999). PPARγ therefore controls key pathways that influence the fate of cells in inflammatory diseases and cancer.

NSAIDs act as fatty acid mimetics (English et al., 1996) and may therefore be expected to interfere with signaling

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; CPA, *cis*-parinaric acid; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated response element; DMSO, dimethyl sulfoxide.

pathways such as the PPAR family of fatty acid-activated transcription factors. Indomethacin, fenoprofen, and ibuprofen activate PPARγ at concentrations of 50 to 500 μM and inhibit the production of monocyte inflammatory cytokines in vitro (Jiang et al., 1998). Such NSAIDs may activate PPARγ at the concentrations achieved during high-dose therapy for rheumatoid disease; however, there have been no reports of NSAIDs binding PPARγ with high affinity or acting as an antagonist of this receptor.

PPARγ is therefore a likely alternative target for NSAID action, but this has not been extensively investigated. We therefore examined a range of NSAIDs for their ability to modulate PPARγ signaling. Here, we show that the widely used analgesic diclofenac binds PPARγ at clinically relevant concentrations and also antagonizes PPARγ *trans*-activation in a receptor-dependent manner, producing distinct physiological effects.

Experimental Procedures

Materials. Unless stated otherwise, chemicals were obtained from Sigma-Aldrich Company Ltd. (Poole, UK) and solvents from BDH Laboratory Supplies (Poole, UK). *cis*-Parinaric acid (CPA) was obtained from Molecular Probes (Leiden, The Netherlands).

Measurement of the Affinity of NSAIDs for PPARγ. The affinity of the NSAIDs fenoprofen and diclofenac for PPARγ was measured using a spectrophotometric binding assay as described previously (Palmer and Wolf, 1998). Briefly, CPA is a ligand for PPARγ and its absorption spectrum changes on binding this receptor. When CPA is displaced from PPARγ by a competitor for the binding site, its spectrum returns to that of the unbound state and the IC_{50} of the competitor can be determined. The magnitude of the spectral shift is quantified by determining the ratio of the absorbencies at 319 nm and 329 nm. The $A_{319\ nm}/A_{329\ nm}$ ratio of CPA in solution is between 2.6 and 3, and the ratio for CPA, when fully bound to the receptor, is 1. *cis*-Parinaric acid was added to 25 mM Tris-HCl, pH 7.5, at room temperature and scanned against buffer between 312 nm and 340 nm, using a UV-3000 scanning spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). Subsequent additions of protein (purified hPPARγ LBD) and competitor were added equally to both sample and reference cuvettes.

When bound to PPARγ, *cis*-parinaric acid produces fluorescence that is diminished when the fatty acid is displaced by a competitor. Therefore, a modified assay can be used in addition to the one described above to determine the K_i values of ligands with an affinity in the nanomolar range. Such an assay was carried out with diclofenac using 200 nM CPA and 250 nM recombinant hPPARγ LBD. These concentrations are well below the K_d value of *cis*-parinaric acid and therefore avoid the spurious results caused by the stoichiometric binding seen when the experiment is performed in the presence of concentrations of protein and *cis*-parinaric acid that are above their K_d values. Fluorescence was measured using an excitation wavelength of 318 nm and emission wavelength of 410 nm with an LS-3 fluorescence spectrophotometer (PerkinElmer Life Sciences, Cambridge, UK). The sum of the fluorescence produced by the protein alone and the CPA alone were deducted from the measured value. The EC_{50} was determined using a single site displacement model and the K_i value was calculated using the equation $K_i = EC_{50} / (1 + [ligand]/K_d)$ (Prism for Macintosh version 3.0a; GraphPad Software, San Diego, CA).

Cell Culture. Dulbecco's modified Eagle's medium (high glucose) supplemented with 9% fetal calf serum, 45 IU/ml penicillin G, and 45 μg/ml streptomycin was used except where stated otherwise. Cells were grown in an atmosphere of 5% carbon dioxide. Cell culture reagents were obtained from Invitrogen (Paisley, UK).

trans-**Activation Assay.** COS-1 cells were seeded in six-well plates (Iwaki Glass Co., Ltd., Funabashi-shi Chiba-ken, Japan) at 3×10^5 per well. The following day they were transfected with an expression vector for hPPARγ (50 ng per well), a luciferase reporter construct (500 ng), and an internal β-galactosidase control vector (500 ng), using a DEAE-dextran method (Cullen, 1987). The vector pCLDN (Brighty et al., 1991) was used to express full-length hPPARγ1 protein. The NSAID activation experiments used the LFAB-P(DR1)$_4$-TK-GL3 reporter vector [containing four copies of the liver fatty-acid binding peroxisome proliferator response element (PPRE) upstream of a luciferase reporter driven by a thymidine kinase promoter], which was a gift from GlaxoSmithKline (Welwyn Garden City, UK). The internal control plasmid, p-SV-β-galactosidase, was obtained from Promega Corporation (Southampton, UK). Drugs (except rosiglitazone, which was a gift of GlaxoSmithKline) were obtained from Sigma-Aldrich, dissolved in DMSO, and added to cell culture medium to give a final DMSO concentration of 0.1% in all experiments. The day after transfection, the cells were exposed to drug for 18 h and underwent lysis the following day. Luciferase expression was quantified by measuring the activity of luciferase with a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA) using luciferase assay reagent (E1501; Promega). The luciferase reading was divided by the β-galactosidase activity (E2000 β-galactosidase enzyme assay system, 96-well format; Promega) from the same replicate to correct for the transfection efficiency.

Adipogenesis Assay. Preadipocytes (3T3-L1) were grown to confluence in an atmosphere of 10% CO_2. Forty-eight hours after confluence, the cells were treated for six consecutive days, changing the medium and drugs every second day. Rosiglitazone, with and without diclofenac, was added, along with 5 μg/ml of insulin (Sigma) and 1 μM dexamethasone (Sigma). DMSO alone was used as a control. The degree of differentiation was examined by staining with the lipid-soluble dye Oil Red O (Sigma) as described previously (Ramírez-Zacarías et al., 1992). Representative areas of staining were photographed and the amount of lipid accumulation was quantified by extracting the dye with isopropanol and measuring the absorbance at 510 nm.

Prostate Cancer Cell Growth Assay. Prostate cancer cells (DU-145) were plated at 2000 cells per well on 24-well plates (Iwaki) in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. After 24 h, the medium was replaced by RPMI 1640 medium without phenol red, supplemented with 5% dextran-charcoal (Sigma) treated fetal calf serum with 2 mM glutamine. Treatments were with 10 μM rosiglitazone, 25 μM diclofenac, or a combination of both drugs. The medium was replaced every second day with fresh drug-containing medium. After 6 days of treatment, cell number was measured by a colorimetric assay based on the activity of lysosomal hexosaminidase (Landegren, 1984).

Statistical Analysis. Data were analyzed using GraphPad Prism. The *trans*-activation assay results in Figs. 2A and 3B were analyzed by one-way ANOVA using Dunnett's post test to compare the results with the DMSO control. ANOVA with no post test was used to analyze the data in Fig. 2B. The Oil Red O extraction data and the results of the prostate cancer growth assay were analyzed by one-way ANOVA, using Tukey's multiple comparison post test to compare the drugs effects with each other and with the control. The significance of the effect of diclofenac on rosiglitazone action was analyzed by unpaired, two-tailed t tests at each dose of rosiglitazone. Statistical significance was defined as a p value of 0.05 or less.

Results

Diclofenac Has an Affinity for PPARγ 50 Times Greater Than Those Reported for Other NSAIDs. PPARγ has been suggested as an alternative target for NSAID action. Therefore, we examined a range of NSAIDs for their ability to modulate PPARγ signaling. To assess the

binding of NSAIDs to PPARγ, we used a spectrophotometric assay examining the displacement of CPA from the ligand-binding domain of PPARγ. Consistent with previous reports, fenoprofen displaced CPA at relatively high concentrations (>100 μM). Surprisingly, diclofenac displaced CPA in a stoichiometric fashion (Fig. 1A); i.e., the *cis*-parinaric acid was present at 1.5 μM in this assay and the diclofenac displaced the *cis*-parinaric acid with an EC_{50} of 1.6 μM. This experiment suggested that the K_i value of diclofenac for PPARγ was below 1.5 μM. The binding of diclofenac to PPARγ was further examined using a fluorescence-based assay, which allowed the use of lower concentrations of *cis*-parinaric acid. These assays demonstrated that diclofenac displaced CPA from PPARγ with a K_i value of 700 nM (Fig. 1B). Therefore, diclofenac has an affinity for PPARγ more than fifty times greater than has been previously reported for an NSAID (Lehmann et al., 1997) and about four times greater than the putative endogenous ligand, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (Forman et al., 1995).

Diclofenac Is a Weak PPARγ Agonist and Antagonizes PPARγ *trans*-Activation. We employed a *trans*-activation assay to measure the effect of diclofenac on the function of the PPARγ receptor. The assay was validated using known PPARγ agonists, which caused activation of the reporter gene at the expected concentrations: rosiglitazone at 500 nM [6.6-fold activation over control, $p < 0.05$] and the NSAID indomethacin at 500 μM [7.5-fold, $p < 0.05$] (Fig. 2A). Despite its relatively high affinity for PPARγ, diclofenac (25 μM) induced only 2-fold activation of the reporter gene (Fig. 2A). In the absence of exogenous PPARγ receptor, increasing

doses of diclofenac induced no reporter gene activation, whereas in the presence of receptor, a significant but small response to the higher doses was seen [$p < 0.05$] (Fig. 2B). Concentrations of diclofenac greater than 30 μM were toxic to the COS-1 cells, causing a progressive decrease in the β-galactosidase activity (data not shown). We then tested whether diclofenac could antagonize the PPARγ agonist rosiglitazone. The dose-response for rosiglitazone was attenuated in the presence of 10 μM diclofenac (Fig. 3A). As would be expected for a competitive partial agonist, this inhibition was overcome at saturating doses of rosiglitazone, with the statistical difference between the two treatments disappearing at 500 nM rosiglitazone. The reporter gene activation by rosiglitazone is PPARγ-specific, as can be seen from the results of the same experiment when carried out using the expression vector pCLDN as a control (Fig. 3A, open symbols). This confirmed minimal reporter gene activity in the absence of exogenous PPARγ. These results show that, unlike indomethacin, which acts as a complete agonist at high concentrations, diclofenac inhibits PPARγ signaling and can significantly reduce reporter gene activation by rosiglitazone. Increasing concentrations of diclofenac caused increasingly significant inhibition of rosiglitazone *trans*-activation (Fig. 3B).

To test whether diclofenac could alter the physiological effects of PPARγ signaling, we used two separate in vitro bioassays of rosiglitazone action. First, we used the 3T3-L1
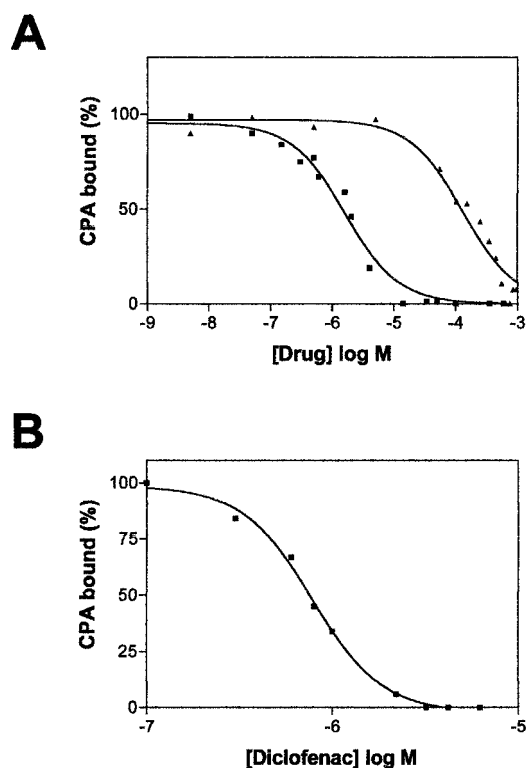

Fig. 1. Diclofenac binds to PPARγ at low concentrations. A, spectral shift binding assay. Diclofenac (■) binds PPARγ at low concentrations, similar to the concentration of protein used in this assay. Fenoprofen (▲) has much weaker affinity. B, fluorescence displacement assay, using lower concentrations of protein. Diclofenac has an IC_{50} of 700 nM.
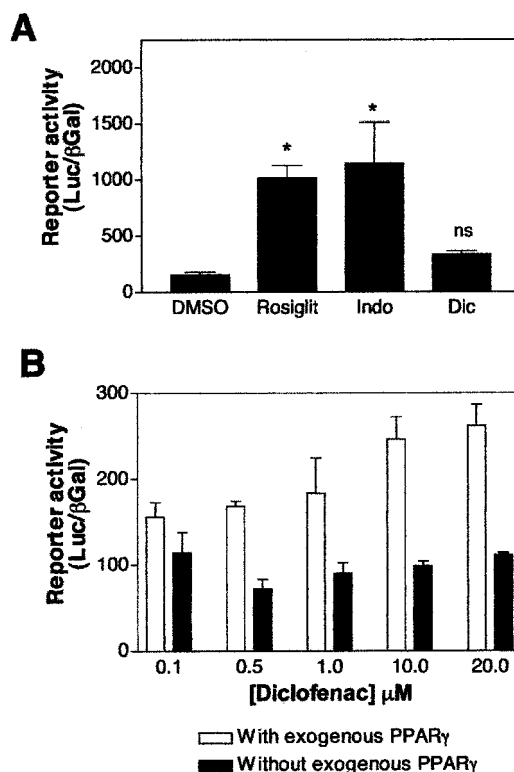

Fig. 2. Diclofenac is a weak activator of PPARγ. A, the effect of different NSAIDs on *trans*-activation of a PPRE-containing reporter construct by PPARγ in COS-1 cells. The effects of 500 nM rosiglitazone (Rosiglit), 500 μM indomethacin (Indo), 25 μM diclofenac (Dic), or DMSO control are shown. Error bars are S.E.M. *, $p < 0.05$; ns, not significant compared with control. B, in the absence of exogenous PPARγ receptor, increasing doses of diclofenac induce no reporter gene activity. In the presence of exogenous receptor, the highest doses of diclofenac induce reporter gene activation of approximately 1.5-fold, $p < 0.05$. Error bars are S.E.M.

preadipocyte cell line in an adipocyte differentiation assay, which is well established in demonstrating the effect of PPARγ agonists. Second, we employed a cell proliferation assay, using a cancer cell line that has been shown previously to be growth-inhibited by rosiglitazone (Mueller et al., 2000).

Diclofenac Inhibits Rosiglitazone-Induced Adipocyte Differentiation. In the 3T3-L1 adipogenesis assay, 100 nM rosiglitazone caused the preadipocytes to become highly differentiated, as shown by the extensive Oil Red O staining of the mature adipocytes (Fig. 4; $p < 0.001$). Treatment with 25 μM diclofenac caused minimal visible differentiation (Fig. 4A), and measurement of lipid accumulation in the diclofenac-treated cells was similar to that of the control ($p > 0.05$; Fig. 4B). However, when preadipocytes were treated with both rosiglitazone and diclofenac (Fig. 4A), there was a 60% reduction in the Oil Red O staining compared with those treated by rosiglitazone alone (Fig. 4B, $p < 0.001$), confirming that diclofenac is an antagonist of rosiglitazone.

Diclofenac Releases Cancer Cells from Rosiglitazone-Induced Proliferation Arrest. To determine whether diclofenac could influence PPARγ-mediated growth inhibition, we investigated its effect on the proliferation of the PPARγ-expressing prostate cancer cell line, DU-145. Ros-

iglitazone (10 μM) inhibited the proliferation of these prostate cancer cells by 90% compared with the control cells ($p < 0.001$), but this PPARγ ligand-induced inhibition could be partially reversed if the same experiment was repeated in the presence of 25 μM diclofenac (Fig. 5). DU-145 cells proliferate three times faster in the presence of both diclofenac and rosiglitazone than in the presence of rosiglitazone alone ($p < 0.01$).

Discussion

Although NSAIDs are known to modulate prostaglandin production by inhibiting cyclooxygenase (COX), there is evidence that they have other mechanisms of action (Elder et al., 1997). Nonsteroidal anti-inflammatory drugs are used extensively for the relief of pain and are widely available throughout the world without prescription. Many people are exposed to the effects of these drugs and understanding what molecular targets they have in addition to cyclooxygenase is important. PPARγ has a role in cell differentiation and apoptosis and has been implicated in the pathophysiology of atherosclerosis, cancer, and diabetes mellitus. Therefore, the interactions of NSAIDs and PPARγ may have clinical consequences in several common human diseases (Jiang et al., 1998).

This work has demonstrated that, relative to other NSAIDs, diclofenac is a high-affinity, partial agonist of PPARγ. NSAIDs such as indomethacin are PPARγ agonists; others, such as diclofenac, may be competitive antagonists and thus inhibit PPARγ signaling Diclofenac has an affinity for PPARγ that is 10 times lower than observed for rosiglitazone (Lehmann et al., 1995) but has an affinity for PPARγ similar to that of pioglitazone (Lehmann et al., 1995) and troglitazone (Camp et al., 2000).

We have found that diclofenac has an IC_{50} for PPARγ more than 50 times lower than previously reported for other NSAIDs. Despite this, diclofenac is not a potent *trans*-activator of PPARγ, even at the plasma concentrations seen after parenteral administration (Nuutinen et al., 1993). The key implication from these observations is that diclofenac given as a single oral dose will achieve serum concentrations (Willis et al., 1981) that will allow interaction with PPARγ, which may result in the displacement of agonists, leading to inhibition of PPARγ signaling. This also suggests that diabetic patients whose blood glucose is controlled by thiazolidinedione drugs, may experience poorer glycemic control if diclofenac is coadministered. However, this hypothesis may be reversed based on the uncertainties about the mechanism of PPARγ action in promoting insulin sensitivity (Miles et al., 2000; Schoonjans and Auwerx, 2000). In addition to inhibiting PPARγ directly, diclofenac may also diminish PPARγ signaling by other mechanisms, such as inhibiting cyclooxygenase (thereby decreasing the availability of endogenous prostanoid ligands that are PPARγ agonists) and by reducing arachidonic acid release and increasing uptake (Scholer et al., 1986). Therefore, it is a paradox that NSAIDs, such as diclofenac, might in fact promote inflammation by blocking the anti-inflammatory pathway of PPARγ by the three mechanisms described above. Interestingly, there is experimental evidence to support this, showing that NSAIDs may have some proinflammatory properties. Such NSAID-induced inflammation can be reduced by concomitant administration of
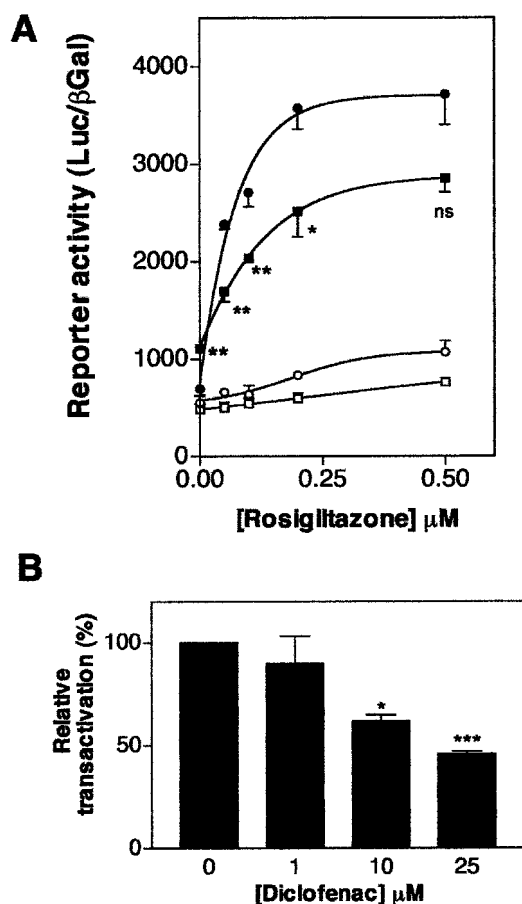

Fig. 3. Diclofenac acts as a partial agonist of PPARγ. A, the effect of 10 μM diclofenac (■) on rosiglitazone-mediated *trans*-activation was compared with that induced by rosiglitazone alone (●). Reporter assays were performed in the presence (filled symbols) or absence (open symbols) of exogenous PPARγ expression. Error bars are SEM. **, $p < 0.01$; *, $p < 0.05$; ns, not significant compared with reporter activity at same dose of rosiglitazone alone. B, increasing concentrations of diclofenac inhibit the reporter gene activation induced by 100 nM rosiglitazone.
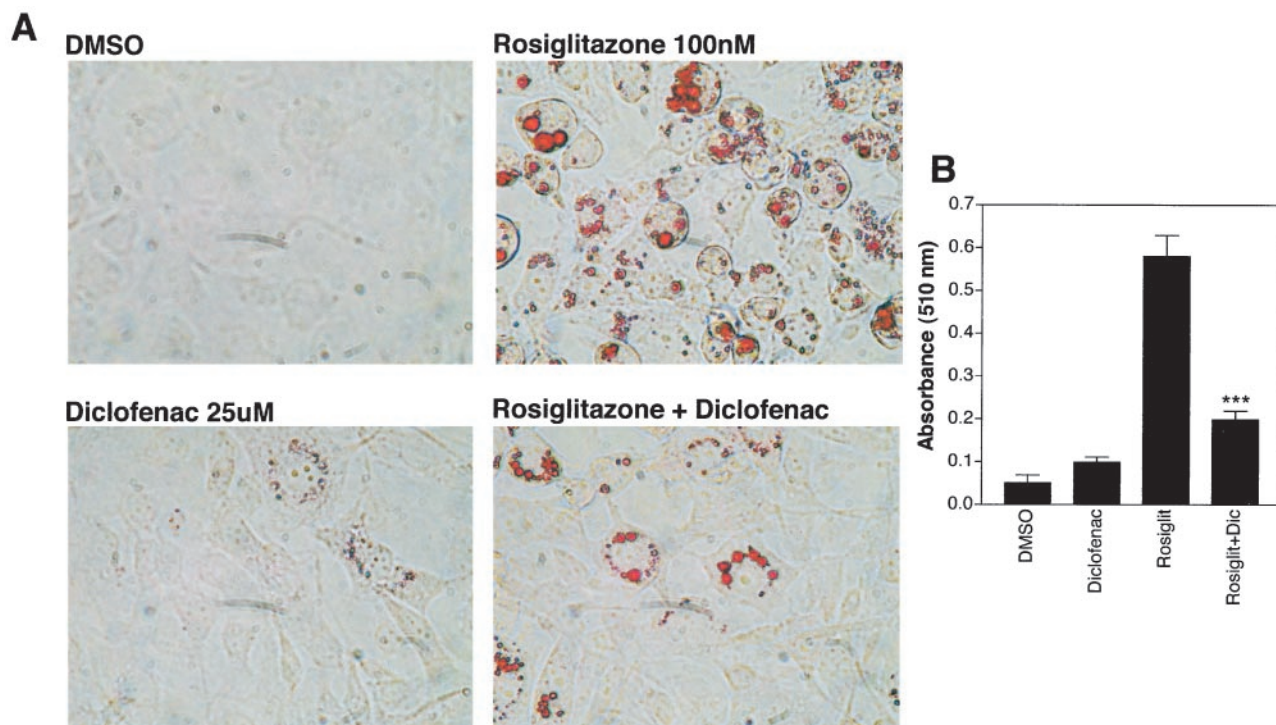
Fig. 4. Diclofenac inhibits rosiglitazone-induced adipogenesis. A, clockwise from top left: DMSO control shows no induction of adipocyte maturation as measured by Oil Red O lipid staining. Rosiglitazone induces extensive adipogenic response, with intense Oil Red O staining indicating maturing adipocytes. Treatment with both diclofenac and rosiglitazone almost completely abolishes the adipogenic action of rosiglitazone alone. Diclofenac is weakly adipogenic, despite being a high-affinity ligand of PPARγ. B, the degree of adipocyte differentiation was determined by quantifying the amount of Oil Red O staining after preadipocytes were treated with DMSO control, diclofenac (Diclofenac), rosiglitazone (Rosiglit), or rosiglitazone + diclofenac (Rosiglit+Dic). Error bars are S.E.M. Treatment with diclofenac and rosiglitazone significantly reduces adipocyte maturation, compared with treatment with rosiglitazone alone; ***, $p < 0.001$.

prostaglandins that are agonists of PPARγ (Gilroy et al., 1999), again pointing to the involvement of this pathway in NSAID action.

Ligands for PPARγ have been used to inhibit the growth of human breast (Mueller et al., 1998), lung (Chang and Szabo, 2000), prostate (Hisatake et al., 2000), and colon (Sarraf et al., 1998) cancer cells. Clinically, PPARγ ligands induce adipocyte differentiation in the tumors of patients with advanced liposarcoma (Demetri et al., 1999). PPARγ is overexpressed (DuBois et al., 1998) and mutated (Sarraf et al., 1999) in colon cancer, suggesting involvement in this carcinogenic process. It is not surprising, therefore, that PPARγ ligands are now being used in clinical trials for the treatment of these common cancers. Interestingly, NSAIDs are also a group of compounds that have been used both experimentally (Rao et al., 1995) and clinically (Giardiello, 1994) as chemopreventative agents in malignant disease, particularly in colon cancer. It is believed that NSAID-mediated effects on cancer cells occur through mechanisms in addition to cyclooxygenase inhibition, and we have shown that NSAIDs may influence cancer cell growth by acting through PPARγ. It seems unlikely that indomethacin or fenoprofen will commonly achieve the plasma concentrations (Lehmann et al., 1997) that would allow significant PPARγ interactions. Conversely, diclofenac may readily achieve the concentration required for receptor binding but may thereby inhibit PPARγ signaling and interfere with PPARγ-mediated growth suppression. Another isoform of PPAR (δ) has also been implicated as a target for NSAIDs in colon cancer (He et al., 1999), and a compound that is structurally related to NSAIDs has

recently been shown to be an irreversible antagonist of PPARα signaling (Kehrer et al., 2001). The PPAR-activating properties of NSAIDs should therefore be considered care-
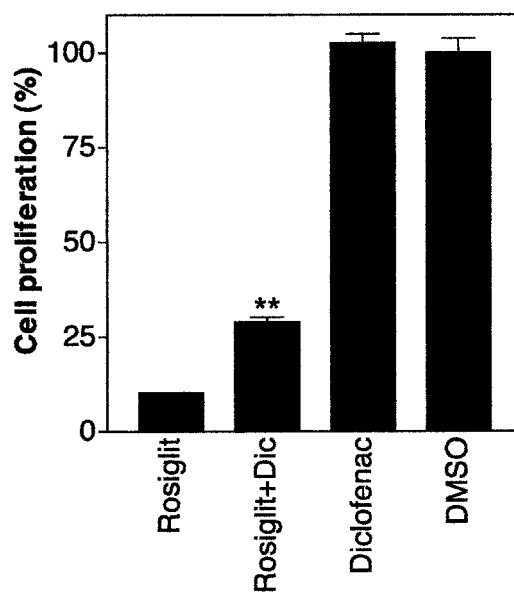

Fig. 5. Diclofenac inhibits rosiglitazone in prostate cancer cells. DU-145 proliferation was measured after treatment with rosiglitazone (Rosiglit), rosiglitazone + diclofenac (Rosiglit+Dic), diclofenac (Diclofenac), or DMSO control. Error bars are SEM. Treatment with diclofenac and rosiglitazone significantly increases cell proliferation, compared with treatment with rosiglitazone alone, **$p < 0.01$.

fully when choosing compounds to be used for cancer chemoprevention studies.

In summary, diclofenac has an affinity for PPARγ 50 times greater than previously reported for other NSAIDs and is a partial agonist that can inhibit the action of the thiazolidinedione rosiglitazone in a receptor-dependent manner. Antagonism of PPARγ signaling by diclofenac prevents adipocyte differentiation and alters the proliferation kinetics of a PPARγ-dependent cancer cell line, releasing the cells from proliferation arrest.

Acknowledgments

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