Inhibition of HER-kinase activation prevents ERK-mediated degradation of PPARγ

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Summary

R-etodolac, a nonsteroidal anti-inflammatory drug, inhibits the progression of CWRSA6 androgen-independent and LuCaP-35 androgen-dependent prostate cancer xenograft growth through downregulation of cyclin D1 expression via the PPARγ pathway. PPARγ protein degradation, observed post-R-etodolac treatment, resulted from phospho-MAP kinase (p44/42) induction by R-etodolac negatively regulating PPARγ function. Negative regulation of PPARγ was overcome by a combination regimen of R-etodolac with the HER-kinase axis inhibitor, rhuMab 2C4, which demonstrated an additive antitumor effect. We further show that the inhibition of HER-kinase activity by rhuMab 2C4 is sufficient to inhibit PPARγ protein degradation. This study introduces a novel concept of an in vivo crosstalk between the HER-kinase axis and PPARγ pathways, ultimately leading to negative regulation of PPARγ activity and tumor growth inhibition.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily and act as ligand-induced transcription factors. Upon ligand stimulation, these receptors form heterodimers with a retinoid X receptor (RXR) and bind to peroxisome proliferator response elements (PPREs), modulating the rate of transcription of target genes (Sporn et al., 2001). Three isoforms of PPAR have been identified, α, δ, and γ, which are encoded by separate genes. PPARγ has been further characterized into three subtypes, $PPAR\gamma_1$, $PPAR\gamma_2$, and $PPAR\gamma_3$, that are derived from distinct transcription start sites followed by alternate splicing events (Fajas et al., 1998; Green, 1995; Zhu et al., 1995). No significant functional difference between the PPARγ variants has been reported; however, the relative expression of these variants is tissue specific. $PPAR\gamma_1$ appears to be expressed in several tissues, while expression of $PPAR\gamma_2$ is primarily restricted to adipocytes (Fajas et al., 1997).

Studies suggesting that PPARγ ligands inhibit cell proliferation while inducing adipocyte differentiation (Tontonoz et al., 1994b) have led to the investigation of the effects of PPARγ activation in various forms of cancer. Activation of PPARγ by pioglitazone, a PPARγ ligand, blocks the cell cycle and causes differentiation of primary liposarcoma cells in culture (Tontonoz et al., 1997). Subsequently, it was demonstrated that treatment of liposarcoma patients with thiazolinediones (TZDs) resulted in retardation of growth and induction of differentiation of the tumor cells (Tontonoz et al., 1997). Troglitazone, a current therapy for the treatment of diabetes, is well established as an antitumorigenic compound, inhibiting the growth of colon, breast, and prostate cancer xenografts (Elstner et al., 1998; Kubota et al., 1998; Sarraf et al., 1998). Such observations have encouraged continued research into the development of novel PPARγ ligands for the treatment of carcinogenesis.

Recently, attention has been focused on the antiproliferative activity of nonsteroidal anti-inflammatory drugs (NSAIDs) in cancerous or transformed cells, which is mediated through an interaction with PPARγ (Na and Surh, 2003). Etodolac is one such novel NSAID (Demerson et al., 1983). Etodolac is pyranocarboxylic acid, chemically designated as (±) 1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-b]indole-1-acetic acid. It exhibits anti-inflammatory, analgesic, and antipyretic activities. The molecular basis for the therapeutic actions of NSAIDs are believed to be through

SIGNIFICANCE

This manuscript demonstrates the clinical utility of R-etodolac, a novel PPARγ transactivator, in prostate cancer xenografts. In addition, the ability of R-etodolac to regulate the levels of PPARγ in a cell through the induction of phospho-MAP kinase via the HER-kinase axis is demonstrated. This is a report of crosstalk between these axes. The clinical utility of this crosstalk is demonstrated by additive growth inhibitory properties of R-etodolac and 2C4, a HER-2-specific monoclonal antibody currently in Phase II clinical trials. Our successful use of this combination treatment regimen demonstrates the potential utility of 2C4 to increase sensitivity of epithelial cancers to PPARγ ligands.

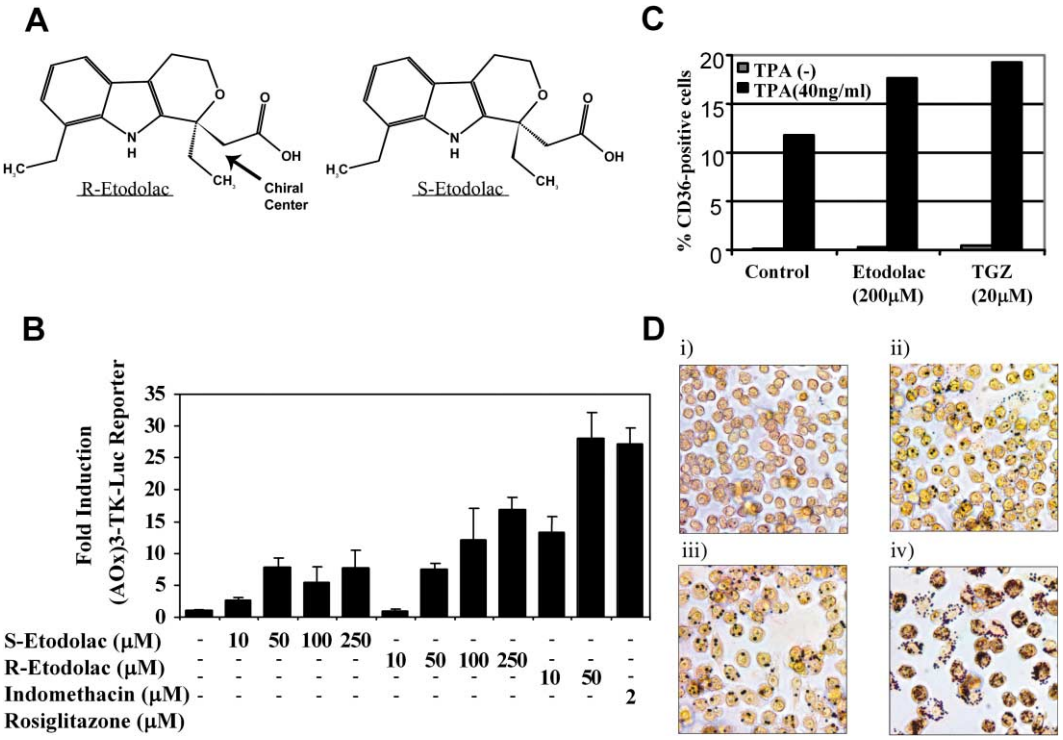

Figure 1. R-etodolac transactivates PPARγ

A: Etodolac is a member of the nonsteroidal anti-inflammatory drug (NSAIDs) family. It is a pyranocarboxylic acid, chemically designated as (±) 1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-b]indole-1-acetic acid. Due to its chiral center (indicated by an arrow), etodolac exists as an enantiomeric mixture of R-etodolac and S-etodolac.

B: Transactivation of a PPARγ reporter construct $(AOx)_3$-TK-Luc by increasing concentrations of etodolac, indomethacin, and rosiglitazone in RAW 267.4 mouse macrophages. Results are presented as a fold-induction of luciferase expression relative to the no drug treatment control and are expressed as the mean ± SD of three separate experiments. Transfection efficiency was normalized using a CMV-β-gal reporter construct.

C: Enhancement of phorbol ester (TPA) induced expression of the CD36 scavenger receptor in THP-1 cells by etodolac and troglitazone (TGZ). Increase in CD36 expression was determined by flow cytometry in presence or absence of TPA with the indicated drugs. Data are presented as the difference in mean fluorescence between anti-CD36 antibody and control isotype-matched antibody.

D: Lipid accumulation in NIH3T3-PPARγ cells induced by R-etodolac. NIH3T3 cells expressing recombinant PPARγ were treated for 7 days with (1) vehicle (DMSO) alone, (2) 1 μM Troglitazone, (3) 1 μM R-etodolac, or (4) 500 μM R-etodolac. The cells were stained for neutral lipids with oil red O stain. The photographs are displayed as a 400× magnification. The dark spots specify the accumulation of neutral lipids.

inhibition of cyclooxygenase (COX) activity, thereby blocking the production of prostaglandins (PGs). However, there is a need to determine the chemopreventative and antimetastatic effects of NSAIDs that can be separated from COX inhibition, because COX inhibition leads to many undesirable side effects (Allison et al., 1992). Due to its chiral center, etodolac exists as an enantiomeric mixture of R- and S-etodolac (Figure 1A). Unlike all other chiral NSAIDs, the two enantiomers of etodolac are not metabolically interconvertible. The etodolac analog, R-etodolac (SDX-101), lacks COX inhibitory activity and is considered the "inactive" enantiomer of this drug (Adachi et al., 2004; Demerson et al., 1983). It was recently demonstrated to inhibit transcription of a β-catenin-dependent T cell and lymphoid enhancing transcription factor (TCF/LEF) reporter gene in HEK293 cells, and at the same concentrations, to diminish the in vitro survival of chronic lymphocytic leukemia (CLL) cells (Lu et al., 2004). R-etodolac is currently being tested in Phase II clinical trials for treating CLL.

PPARγ is significantly expressed in primary prostate cancer but at very low levels in normal prostate tissue, making it a promising candidate for molecular therapy in prostate cancer (Segawa et al., 2002). Hemizygous deletion of PPARγ is common in primary human prostate cancers, suggesting that loss of function may contribute to the malignant phenotype (Mueller et al., 2000). In accordance with this observation, PPARγ activation with synthetic ligands downregulates prostate-specific antigen (PSA) mRNA expression in prostate cancer cells in vitro (Hisatake et al., 2000; Kubota et al., 1998). Moreover, a phase II clinical study with troglitazone in patients with prostate cancer was associated with prolonged periods of stable disease characterized by the absence of new metastases or disease-related symptoms (Mueller et al., 2000).

The HER-kinase signaling network is active in prostate cancer cells and is known to contribute significantly to the progression of the disease (Agus et al., 2002). The human epidermal growth factor receptor (HER) or ErbB family of receptor tyrosine kinases (RTKs) are important mediators of cell growth, survival, and differentiation. The family consists of four distinct receptors, HER1, HER2, HER3, and HER4 and at least 11 known ligands that have specificity to these receptors (Yarden and Sliwkowski, 2001). The signaling network is activated by receptor-specific ligand stimulation that leads to receptor dimerization and auto-

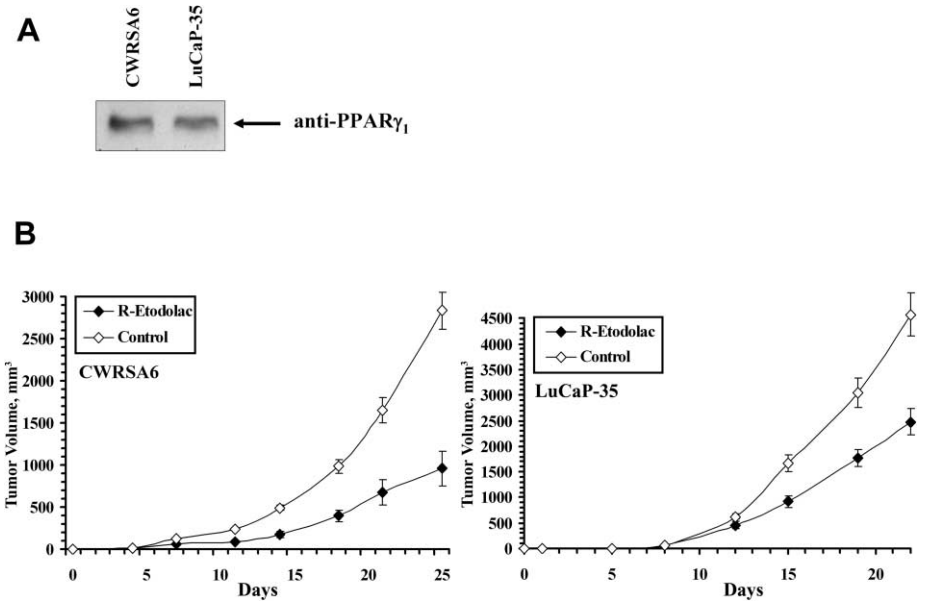

Figure 2. Effect of R-etodolac on PPARγ1-positive prostate cancer xenografts

A: Representative Western blot analysis showing PPARγ1 expression in protein lysates prepared from androgen-independent CWRSA6 and androgen-dependent LuCaP-35 human prostate cancer xenografts.

B: The response of CWRSA6 or LuCaP-35 tumors to R-etodolac (closed diamond) administered at a dosage of 200 mg/kg via daily o.g. or vehicle-treated control (open diamond). Therapy was initiated on day 6 in the CWRSA6 tumor line and on day 9 in the LuCaP-35 tumor line. Results are presented as mean tumor volume (n = 10) ± SE.

phosphorylation. Therapies directed against the HER-kinase axis, such as rhuMab 2C4 (Agus et al., 2002), inhibit the growth of prostate xenografts by as much as 80%, corroborating the concept that the HER-kinase axis is an important mediator of prostate cancer growth. In vivo studies have demonstrated that ligands for the HER-kinase axis induce phosphorylation of PPARγ1 by mitogen-activated protein (MAP) kinase (Camp and Tafuri, 1997), which may eventually regulate the levels of PPARγ. However, none of the studies have suggested a direct modulation of the HER-kinase axis by a PPARγ activator. The hypothesis for the crosstalk between the HER-kinase axis and the PPARγ pathway seems plausible since the former is overexpressed to appreciable levels in prostate cancer.

In this study, we report the mechanism of action of R-etodolac in inhibiting tumor growth of two prostate cancer xenograft models, i.e., the androgen-independent CWRSA6 and the androgen-dependent LuCaP-35. R-etodolac exhibits PPARγ-specific activity and has the putative ability to modulate the HER-kinase axis. Treatment with R-etodolac leads to the induction of phospho-MAP kinase activity via the HER-kinase axis, resulting in the degradation of the PPARγ protein. Combination therapy with HER-kinase inhibitor rhuMab 2C4 (Agus et al., 2002) inhibited R-etodolac-induced activation of MAP kinase, ultimately preventing the degradation of PPARγ protein, thereby increasing the antitumor efficacy of the PPARγ transactivator in prostate cancer xenografts. This study highlights a novel crosstalk mechanism between the PPARγ pathway and the HER-kinase axis that cooperates in prostate tumor growth and further demonstrates that this crosstalk can be successfully inhibited with a drug combination therapy targeting these signaling pathways.

Results

R-etodolac transactivates PPARγ

We used three different assays to demonstrate that R-etodolac has the ability to function as a PPARγ transactivator. (1) R-etodolac enhances reporter gene expression in a dose-dependent manner driven by promoters containing PPRE sequences in transient transfection assays (Figure 1B). This transactivation function is mediated only in the presence of recombinant PPARγ. The COX inhibitory analog of etodolac, S-etodolac, can also transactivate PPARγ but to significantly lower levels. Transactivation by 100 μM R-etodolac (13-fold) is comparable to 10 μM of the known PPARγ ligand and NSAID, indomethacin, suggesting that R-etodolac has PPARγ transactivation activity. Rosiglitazone, another demonstrated PPARγ ligand, was used as a positive control. (2) The combination of PPAR-γ transactivation, together with protein kinase C activation by a phorbol ester, has been reported to induce the scavenger receptor CD36 in macrophages (Han and Sidell, 2002). CD36 is documented as a PPARγ-regulated gene (Tontonoz et al., 1998). Using CD36 expression as a downstream indicator, 200 μM of racemic etodolac and 20 μM of troglitazone (a demonstrated PPAR-γ activator) displayed equivalent potency as measured by a flow cytometric assay (Figure 1C). (3) PPARγ is accepted as a master regulator of adipocyte differentiation. Uptake of neutral lipids is a marker of adipocyte differentiation, and oil red O staining of these neutral lipids is an accepted procedure to demonstrate this differentiation phenomenon (Tontonoz et al., 1998). NIH3T3 cells that stably overexpress retrovirally expressed recombinant PPARγ were obtained from Dr. Ronald Evans (Salk Institute, La Jolla, CA). Cells treated with 1 μM R-etodolac displayed accumulation of neutral lipids and morphological changes associated with PPARγ activity that are comparable to those observed with troglitazone at a similar concentration (Figure 1D). The lipid uptake was dose dependent and was significantly more pronounced at 500 μM concentration of R-etodolac. NIH3T3 cells transfected with the empty retroviral vector did not demonstrate the lipid uptake with either troglitazone or R-etodolac (data not shown). Having demonstrated that PPARγ could be transactivated by R-etodolac, the possibility of using it as a potential therapeutic against prostate tumor models was considered.

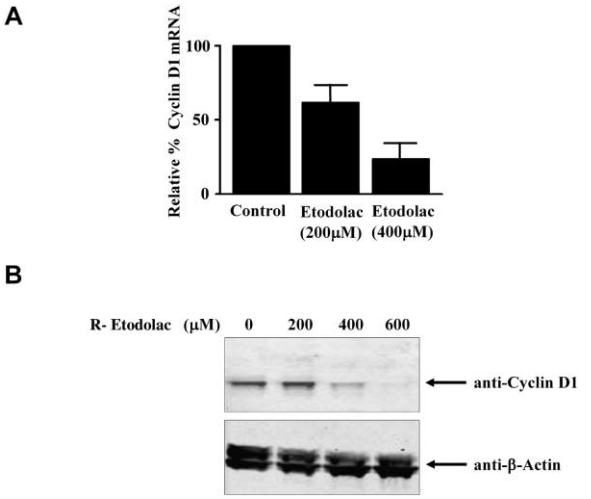

Figure 3. Reduction in cyclin D1 mRNA and protein expression in LNCaP cells treated with R-etodolac

A: Real-time quantitative RT-PCR assay demonstrating cyclin D1 mRNA expression in LNCaP cells treated with increasing concentrations of R-etodolac (0, 200, and 400 μM) for 18 hr. The levels of RNA were normalized using an assay for 18S RNA. The level of cyclin D1 transcripts in untreated cells was set to 100 percent. This graph represents the mean from three independent experiments ± SD.

B: Western blot analysis demonstrating cyclin D1 protein expression in LNCaP cells treated with increasing concentrations of R-etodolac (0, 200, 400, and 600 μM) for 18 hr. β-actin protein is used as a normalization control. This figure is representative of at least 3 independent assays.

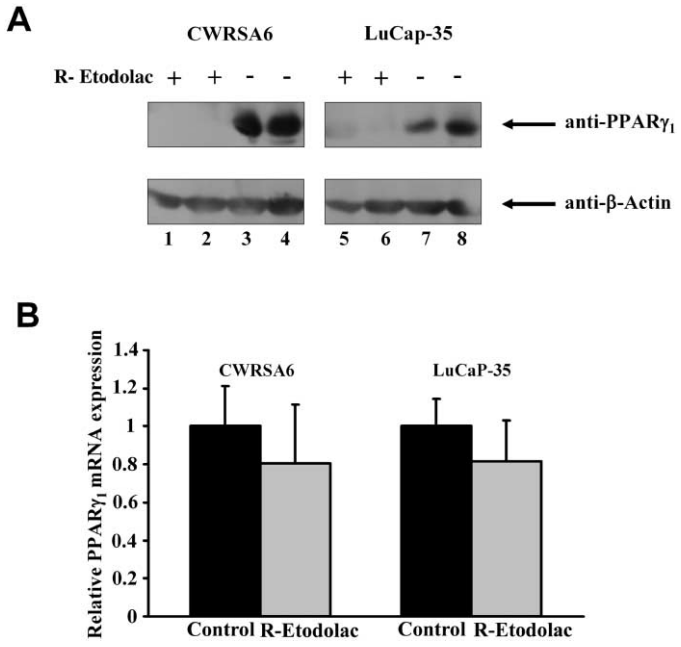

Figure 4. Degradation of PPARγ protein following R-etodolac treatment in CWRSA6 and LuCaP-35 prostate cancer xenografts

A: Western blot analysis demonstrating $PPAR\gamma_1$ expression in protein lysates prepared from the R-etodolac efficacy studies involving the CWRSA6 and LuCaP-35 human prostate cancer xenografts. Lanes 3, 4, 7, and 8 represent vehicle-treated control tumors, whereas lanes 1 and 2 represent CWRSA6 tumors following 19 days of treatment with R-etodolac, and lanes 5 and 6 represent LuCaP-35 tumors following 13 days of treatment with R-etodolac. Equal protein loading was confirmed with a β-actin antibody.

B: Real-time quantitative RT-PCR analysis of $PPAR\gamma_1$ mRNA rexpression in CWRSA6 and LuCaP-35 xenografts following the animal efficacy studies. Levels of PPARγ mRNA expression were normalized to β-actin mRNA levels, and results are displayed as an average (n = 5) relative percentage to control animals ± SD. No significant difference was observed between treated and nontreated control animals.

R-etodolac, a PPARγ transactivator, inhibits the growth of CWRSA6 and LuCaP-35 prostate cancer xenografts

Immunoblot analyses demonstrated the presence of $PPAR\gamma_1$ in the androgen-dependent LuCaP-35 and androgen-independent CWRSA6 xenografts (Figure 2A). Real-time RT-PCR further confirmed the presence of PPARγ mRNA in the xenografts (data not shown). Primers specific for $PPAR\gamma_2$ did not yield an appreciable amplification, whereas total PPARγ-specific primers led to a robust amplification, suggesting that the predominant isoform in the prostate cancer xenografts was $PPAR\gamma_1$. To examine the effect of R-etodolac treatment on tumor cell growth, animals with established androgen-independent CWRSA6 or androgen-dependent LuCaP-35 prostate cancer xenografts were administered R-etodolac (200 mg/kg o.g. daily). At this dose, no lethal toxicity or weight loss (greater than 10% body weight) was observed among treated animals, and a drug concentration of 500 μM was achieved in the serum (data not shown). R-etodolac demonstrated significant tumor growth inhibition in the androgen-independent CWRSA6 xenografts (66% growth inhibition, $p = 0.002$, $n = 10$) and androgen-dependent LuCaP-35 xenografts (46% growth inhibition, $p = 0.0103$, $n = 10$) relative to control-treated animals (Figure 2B). These studies demonstrate that R-etodolac inhibits the growth of PPARγ-positive prostate cancer xenografts.

R-etodolac causes a downregulation in cyclin D1 expression

PPARγ transactivation is well documented to have an effect on cell cycle progression through the repression of the cyclin D1 promoter (Qin et al., 2003; Wang et al., 2003). Accordingly, we observed a downregulation in cyclin D1 mRNA (Figure 3A) and protein (Figure 3B) expression following 18 hr of R-etodolac treatment of LNCaP prostate cancer cells. The decrease in cyclin D1 expression was dependent upon increasing concentrations of R-etodolac. The inhibition of cyclin D1 expression by R-etodolac provides insight into the mechanism by which R-etodolac inhibits tumor growth.

R-etodolac treatment leads to a degradation of PPARγ protein

PPARγ protein levels were analyzed in prostate xenografts following R-etodolac treatment to elucidate the growth-inhibitory mechanism of R-etodolac on prostate xenografts. Since transient transfection studies indicated that R-etodolac serves as a PPARγ transactivator, we expected an upregulation or no change in PPARγ protein since the drug had caused a significant reduction in tumor growth. To our surprise, the PPARγ protein was completely eliminated from the tumors following 2–3 weeks of drug treatment as compared to the vehicle-treated control (Figure 4A). Real-time RT-PCR analysis of $PPAR\gamma_1$ mRNA at this time point showed that expression was similar to that in the control animals (Figure 4B), indicating that PPARγ downregulation was predominantly a posttranscriptional event.

We analyzed phospho-ERK1 and phospho-ERK2 levels fol-

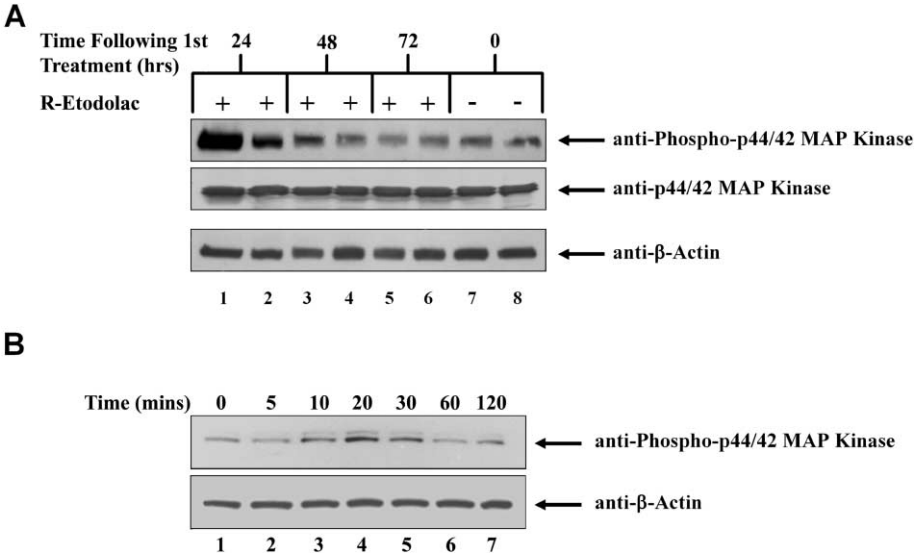

Figure 5. R-etodolac treatment increases phospho-ERK activity in CWRSA6 xenografts and 22Rv1 prostate cancer cells

A: Western blot analysis of lysates prepared from R-etodolac-treated CWRSA6 xenografts following the time course experiment as described in the Experimental Procedures. Animals received R-etodolac every 24 hr; lanes 1 and 2 represent tumors following 24 hr of treatment, lanes 4 and 5 represent tumors following 48 hr of treatment, and lanes 5 and 6 represent tumors following 72 hr of treatment with R-etodolac. Lanes 7 and 8 represents vehicle-treated control animals following 72 hr of treatment. Equal loading was confirmed with a β-actin antibody, as well as with an antibody against total MAP kinase.

B: Whole-cell lysates were prepared from 22Rv1 cells treated with R-etodolac (500 μM) for various times shown. This was a representative experiment repeated independently three times.

lowing R-etodolac treatment since it has been reported that PPARγ phosphorylation by MAP kinase leads to negative regulation of the nuclear receptor's function via the ubiquitin proteosome-mediated degradation pathway (Floyd and Stephens, 2002). Using a phospho-specific MAP kinase antibody, we observed a sharp increase in phosphorylated p44/42 levels 24 hr post-R-etodolac treatment (Figure 5A). This transient increase was followed by a low level of sustained activation of MAP kinase throughout the rest of the time course up to 72 hr. At 3 weeks post drug treatment, immunoblot analysis of phospho-ERK1/2 protein appeared similar to that at 72 hr (data not shown). No difference was observed in total MAP kinase expression, and equal loading was confirmed with an anti-β-actin antibody. These results in the prostate cancer xenograft were later confirmed in vitro by treating 22Rv1 prostate cancer cells with a concentration of R-etodolac (500 μM) physiologically similar to that achieved in the xenograft studies. A transient peak in phospho-MAP kinase activation, although modest as compared to the in vivo studies, was observed within 20 min of the addition of R-etodolac and then maintained a sustained level of activation (Figure 5B). Such transient upregulation in phospho-MAP kinase activation, also observed with troglitazone treatment, ultimately led to degradation of PPARγ protein (Baek et al., 2003).

These results suggest that R-etodolac (200 mg/kg o.g. daily) treatment exerts two opposing effects on tumor growth. Not only does R-etodolac increase the antitumorigenic potential of PPARγ, as demonstrated in the transient transfection studies, but it may also regulate the levels of PPARγ protein via upregulation of phospho-ERK1/2. The latter effect may have a consequence on the antitumorigenicity of R-etodolac.

Combined treatment with R-etodolac and rhuMab 2C4 causes an increased suppression of prostate tumor growth via inhibition of phospho-ERK1/2

Since treatment with R-etodolac resulted in an upregulation of phospho-MAP kinase and subsequent degradation of PPARγ protein, we reasoned that inhibiting phospho-MAP kinase should increase the efficacy of R-etodolac. To prevent MAP kinase activation and subsequent PPARγ degradation, we treated CWRSA6 xenografts with a regimen of R-etodolac in combination with rhuMab 2C4. rhuMab 2C4 (Agus et al., 2002) is a humanized monoclonal antibody that abrogates MAP kinase activation by sterically inhibiting ligand-induced heterodimerization of HER2 with members of the HER-kinase receptor family.

Mice bearing established CWRSA6 xenografts received monotherapy with either R-etodolac (200 mg/kg o.g. daily, $n = 10$) or 2C4 (20 mg/kg i.p. 2×/wk, $n = 10$) or a combination regimen of R-etodolac and 2C4 for 3 weeks ($n = 10$). At these doses, no lethal toxicity or significant weight loss was observed among treated animals. CWRSA6 xenografts receiving a combination regimen of R-etodolac and 2C4 demonstrated significant tumor inhibition compared to animals receiving R-etodolac monotherapy (tumor inhibition 59%, $p < 0.001$, $n = 10$) and 2C4 alone (tumor inhibition 51%, $p = 0.001$, $n = 10$) (Figure 6A). These results support the hypothesis that the efficacy of R-etodolac can be improved by inhibiting phospho-MAP kinase.

One prediction in accordance with our hypothesis would be the rescue of PPARγ protein from degradation following the combination treatment. To ascertain this, total cell lysates were prepared from xenografts in the preceding study and were subjected to immunoblot analysis for PPARγ protein. As expected, samples treated with R-etodolac alone had a complete ablation of the PPARγ protein (Figure 6B, lanes 1 and 2), and animals receiving a combination regimen of R-etodolac and 2C4 maintained their PPARγ protein expression (Figure 6B, lanes 5 and 6). Xenografts treated with 2C4 alone also expressed PPARγ (Figure 6B, lanes 3 and 4). R-etodolac treatment did not alter the mRNA expression of the HER-kinase receptors (data not shown). Based on our observed results, we propose a model for the suggested modes of action of R-etodolac as illustrated in Figure 7.

Discussion

The transcription factor PPARγ has been described as an important antitumorigenic molecule involved in the control of cell growth and differentiation (Tontonoz et al., 1994a). Accordingly, PPARγ ligands, troglitazone, rosiglitazone, and 15-deoxy-

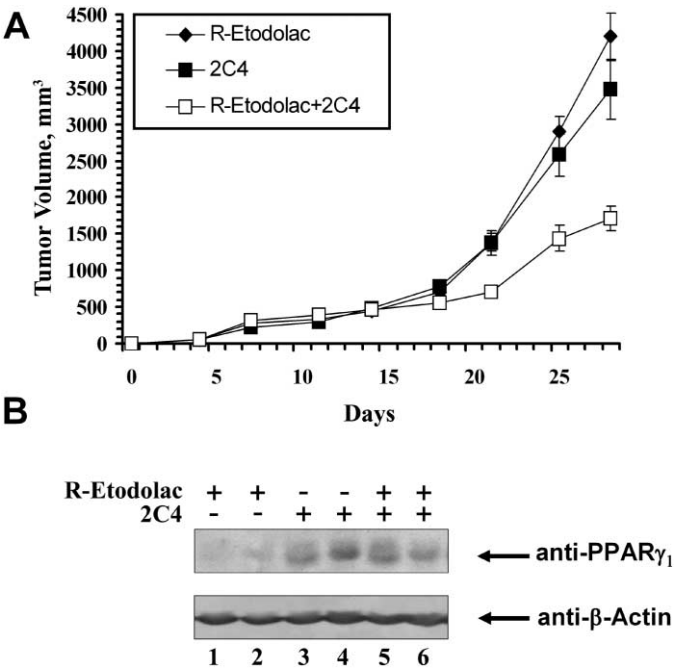

Figure 6. Inhibition of MAP kinase by 2C4 prevents R-etodolac-induced degradation of PPARγ protein, thus promoting efficacy of R-etodolac

A: The response of CWRSA6 tumors to R-etodolac (closed diamond) administered at 200 mg/kg via daily o.g., or 2C4 (closed square) administered at 20 mg/kg via interperitoneal injection, or a combination regimen of R-etodolac and 2C4 (open square) at the previously stated doses. Therapy initiated on day 6. Results are presented as mean tumor volume (n = 10) ± SE.

B: Western blot analysis showing PPARγ$_1$ expression in lysates prepared from the R-etodolac and 2C4 combination efficacy studies involving the androgen-independent CWRSA6 human prostate xenograft tumors following 22 days of treatment. Lanes 1 and 2 represent R-etodolac-treated tumors, lanes 3 and 4 represent tumors receiving 2C4 alone, and lanes 5 and 6 represent tumors receiving a combination of R-etodolac and 2C4.

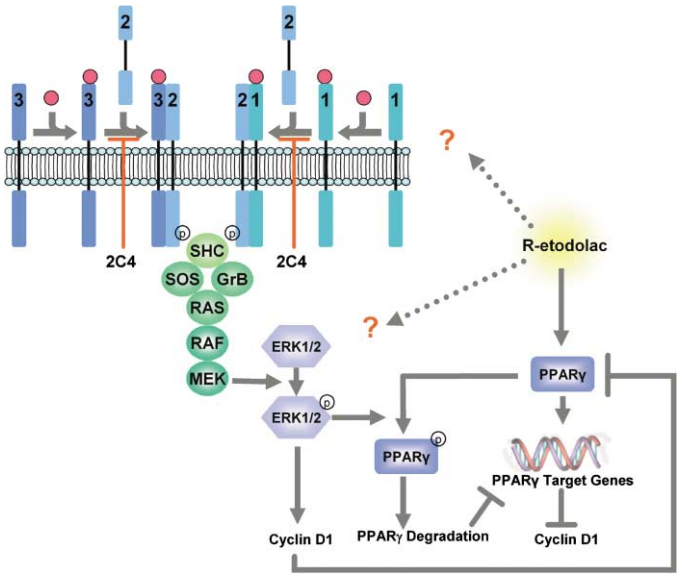

Figure 7. Proposed model of R-etodolac action

R-etodolac transactivates PPARγ activity, thereby modulating expression of genes contributing to tumor growth inhibition. This transactivation decreases cyclin D1 expression, which aids in tumor growth. R-etodolac treatment, while decreasing cyclin D1 expression via the PPARγ pathway, also induces cyclin D1 via phospho-MAPK (Lavoie et al., 1996). Cyclin D1 is known to repress PPARγ expression and transactivation (Wang et al., 2003). Phospho-MAPK induction by R-etodolac may or may not be a PPARγ-dependent effect. Phospho-MAPK phosphorylates PPARγ, leading to an inactivation of PPARγ transcriptional activity due to decreased ligand affinity and unbiquitin-mediated proteosome degradation of the PPARγ receptor (Camp and Tafuri, 1997). It is unknown how R-etodolac stimulates phospho-MAPK activity; however, the additive antitumor effect of 2C4 and R-etodolac implicates the involvement of the HER-kinase axis as a possible activator of phospho-MAPK. The numbers 1, 2, 3, and 4 refer to the HER-kinase receptors HER-1, HER-2, HER-3, and HER-4, respectively. Red circles refer to ligands that can stimulate the HER-kinase receptors.

Δ-12,14-prostaglandin J_2 have demonstrated growth inhibition of prostate cancer cell lines that express an appreciable level of PPARγ (Mueller et al., 2000; Segawa et al., 2002). Furthermore, treatment of prostate cancer patients with troglitazone has been shown to lower serum PSA levels (Mueller et al., 2000).

Here we describe a PPARγ transactivator, R-etodolac. R-etodolac is a stable stereoisomer of an approved nonsteroidal anti-inflammatory drug lacking cyclo-oxygenase inhibitory activity that can effectively suppress the growth of prostate cancer xenograft models without apparent morbidity. Our study demonstrates that R-etodolac displays a potent transactivation activity in the presence of PPARγ in cell culture-based transient transfection assays. This activity is dose dependent and is comparable to the established NSAID PPARγ agonist, indomethacin. We support the notion that R-etodolac is a PPARγ transactivator by using CD36 expression and the uptake of neutral lipids as a marker of adipocyte differentiation in the presence of R-etodolac and PPARγ. In accordance with these observations, o.g. administration of R-etodolac led to a significant reduction in established tumor volumes of PPARγ-expressing androgen-dependent and androgen-independent tumors.

We chose two xenograft models for our studies, CWRSA6 and LuCaP-35, since these models have been well characterized in terms of PSA progression, androgen receptor status, and dependence on HER-kinase axis. These models represent two distinct stages of prostate cancer, i.e., the androgen-dependent (LuCaP-35) stage and the androgen-independent (CWRSA6) stage (Corey et al., 2003; Nagabhushan et al., 1996). The activity of R-etodolac did not appear to distinguish between the androgen-dependent and -independent models in terms of its efficacy. Androgens play a critical role in prostate cancer survival and progression, which is why androgen ablation therapy is the accepted first line of treatment for metastatic disease. Prostate cancer subsequently progresses to an androgen-independent state, the underlying mechanisms for which are not clear. We focused our subsequent studies on the androgen-independent model since there is an urgent need in the field to identify new therapies to treat this cancer state.

We propose that the primary mechanism of antitumor activity exerted by R-etodolac is through the enhancement of transcriptional activity of the PPARγ receptor. PPARγ is well documented to have an effect on cell cycle progression through the repression of the cyclin D1 promoter (Wang et al., 2001). Cyclin D1 determines the rate of progression of mammary epithelial cells through the G1 phase in response to mitogenic and oncogenic signals (Lee et al., 2000). Accordingly, we observed a

downregulation in cyclin D1 gene expression following R-etodolac treatment of prostate cancer cell lines. R-etodolac may also contribute to tumor inhibition via upregulation of apoptosis via the Wnt/β-catenin pathway (Lu et al., 2004).

Following 2–3 weeks of treatment with R-etodolac, xenografts exhibit PPARγ protein loss. This loss of PPARγ protein is a posttranscriptional event because no change in PPARγ mRNA expression was observed. This lack of PPARγ protein may be explained either through posttranscriptional degradation of PPARγ or through selection against PPARγ-positive cells. Selection against PPARγ-positive cells is ruled out by the results of the combination drug therapy study discussed later. It may be tempting to conclude that the antitumorigenic effect of R-etodolac works through loss of PPARγ protein, but this is not the case. PPARγ protein loss as a mechanism of R-etodolac function is refuted by immunoblots of the time course experiment that reveal an upregulation of the PPARγ receptor in the majority of the animals 24 to 72 hr post-R-etodolac treatment (data not shown). The time period of PPARγ upregulation in xenografts receiving R-etodolac coincides with the peak in PPARγ transcriptional activity in cell culture-based assays following R-etodolac administration. These results strengthen the idea that the initial effect of R-etodolac is the increase in PPARγ activity. Our hypothesis is that the observed loss of PPARγ protein is subsequent to PPARγ activation since an essential requirement for PPARγ degradation is the ligand-induced conformational change of the receptor, which is associated with transcriptional activation (Hauser et al., 2000).

R-etodolac has the unique ability to regulate the levels of PPARγ receptors expressed in a cell. This is an important concept since increased transcriptional activity of PPARγ under certain circumstances has been reported to enhance colon cancer progression (Saez et al., 1998). A well-documented mechanism utilized to negatively regulate PPARγ transcriptional activity is via phosphorylation of PPARγ by MAP kinase (Camp and Tafuri, 1997; Floyd and Stephens, 2002; Hauser et al., 2000). This phosphorylation event reduces ligand binding affinity of PPARγ and leads to a downregulation of the PPARγ protein levels by ubiquitin proteosome-mediated degradation (Floyd and Stephens, 2002; Shao et al., 1998). Phosphorylation has also been documented for regulation of the progesterone receptor (Lange et al., 2000; Shen et al., 2001). Our observation that treatment with R-etodolac leads to an upregulation of phospho-MAP kinase and a subsequent loss of PPARγ protein suggests a similar mechanism of regulation. We hypothesize that the loss of PPARγ protein, a target of R-etodolac, may be a mechanism of "autoresistance" to the drug. The loss of PPARγ protein would also compromise the ability of R-etodolac to repress cyclin D1. Additionally, induction of phospho-MAP kinase by R-etodolac would lead to an increase in cyclin D1 since MAP kinase induces the cyclin D1 promoter through a phospho-PPARγ-independent mechanism (Wang et al., 2001; Watanabe et al., 1998). Increased cyclin D1 is documented to repress PPARγ expression and transactivation, limiting its antitumorigenic potential (Wang et al., 2003). These events would add to the mechanism of autoresistance of R-etodolac.

The autoresistance phenomenon can be overcome by use of a combination regimen of R-etodolac and 2C4. The combination regimen promotes maintenance of PPARγ protein in the xenograft, which correlates with increased antitumorigenicity. Treatment with 2C4 in combination with R-etodolac would also perpetuate the inhibition of cyclin D1 through the maintenance of the PPARγ protein and through the abrogation of phospho-MAP kinase activity, thus overcoming the autoresistance to R-etodolac. The combination study also supports the notion that the absence of PPARγ expression in R-etodolac-treated xenografts is not the result of selection against PPARγ-positive cells. Taken together, these observations suggest that R-etodolac-mediated growth inhibition is achieved through activation of the PPARγ receptor while controlling this activity through a mechanism of autoresistance.

Based on the results of the combination therapy with the HER-kinase-specific antibody, 2C4, our study suggests that R-etodolac activates phospho-MAP kinase through stimulation of the HER-kinase axis. It is not known if this is a PPARγ-dependent event. Nevertheless, this observation reveals an important crosstalk between the HER-kinase axis and PPARγ pathway in prostate tumorigenesis. The HER-kinase axis is a positive regulator of prostate cancer cell survival, as demonstrated by growth inhibition curves of prostate xenografts (80%) by the HER2-specific mAb, 2C4 (Agus et al., 2002). In our study, 2C4 growth inhibition is additive to that achieved with R-etodolac alone, and it also rescues PPARγ protein from degradation. These studies suggest the involvement of the HER-kinase axis in PPARγ degradation and also support the proposed model of autoresistance of R-etodolac. Other studies have shown that HER2 mRNA overexpression enhances the expression of PPARγ (Yang et al., 2003); however, we did not observe any change in HER2 receptor levels. While R-etodolac treatment did not alter the mRNA levels of HER-kinase receptors (data not shown), it is possible that the PPARγ transactivator renders the receptors constitutively active or increases the concentration of endogenous ligands. Activation of MAP kinase by ligands such as epidermal growth factor and platelet-derived growth factor induce the phosphorylation of $PPAR\gamma_1$ on Ser82 and decrease its ability to activate transcription (Camp and Tafuri, 1997). In light of these results, we propose a potential model (Figure 7) for the regulation of PPARγ by R-etodolac in prostate cancer xenografts. More experiments will be needed to further elucidate the mechanism by which R-etodolac activates the HER-kinase axis.

An intriguing observation in our study has been that neutral lipid uptake, but not transactivation of PPARγ or tumor growth inhibition, was seen at lower doses (1 μM) of R-etodolac. It is known that modulation of lipid-related genes by PPARγ transactivators occur at several log concentrations lower, closer to the K_d of PPARγ, than the concentrations required to produce anticancer activity or inhibit cytokines via PPARγ (Koeffler, 2003). The concern still remains regarding the high R-etodolac dosage required to see a visible effect on tumor growth inhibition. The high dosage required can be explained due to the autoresistance phenomenon observed with R-etodolac. Autoresistance limits the ability of R-etodolac to cause an effective decrease in cyclin D1 expression via PPARγ. Therefore, a dose increase of the drug may be required to overcome autoresistance to R-etodolac by shifting activity of the system toward the PPARγ-dependent pathway and to observe a net effect on tumor inhibition, since the ubiquitination and proteosome-mediated degradation of PPARγ will become a limiting step at some concentration of R-etodolac, thus increasing the availability of the drug target in the tumors (PPARγ) and an increased antitumorigenic effect. However, an important point to remember

is that R-etodolac does not have COX-inhibitory activity and can accumulate to high levels in the plasma without causing any inflammation-induced damage to internal organs in nude mice.

In summary, this study demonstrates tumor growth inhibition by R-etodolac via a PPARγ-dependent pathway and highlights a crosstalk mechanism between PPARγ and HER-kinase signaling pathways in prostate tumorigenesis.

Experimental procedures

Tumor models

The xenograft studies were performed as previously described (Agus et al., 1999a, 1999b). 4- to 6-week-old nude mice were obtained from National Cancer Institute-Charles Rivers Laboratories and maintained in pressurized ventilated caging at the Cedars-Sinai Medical Center vivarium. Male animals were subcutaneously inoculated with minced tumor tissue from the androgen-dependent LuCaP-35 xenografts (Corey et al., 2003), and females received the androgen-independent CWRSA6 xenografts that were obtained by selecting tumors for regrowth and increased serum PSA after androgen withdrawal (Agus et al., 1999a). All lines were injected together with reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA) and RPMI 1640 supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin as described previously (Nagabhushan et al., 1996; Wainstein et al., 1994). To maintain serum testosterone levels (for the LuCap-35 line), mice were subcutaneously implanted with 12.5 mg sustained release testosterone pellets (Innovative Research of America, Sarasota, FL) 1 week before receiving the tumor cell inoculation. For single agent efficacy studies, treatments consisted of daily oral gavage (o.g.) of 200 mg/kg R-etodolac (Salmedix, Inc.) in water, supplemented with 0.5% Methycellulose and 0.5% Polysorbate 80. The combination regimen study consisted of daily o.g. of 200 mg/kg R-etodolac and twice weekly intraperitoneal (i.p.) injection of 20 mg/kg 2C4 (Genentech, South San Francisco, CA) in phosphate-buffered saline. Tumors were measured every 3–4 days with vernier calipers, and tumor volumes were calculated by the formula $\pi/6 \times$ (larger diameter) $\times$ (smaller diameter)^2. Animals with palpably established tumors of at least 65 mm^3 were designated to treatment groups.

A time course study was implemented using R-etodolac-treated CWRSA6 xenografts. 14-day-old CWRSA6 xenografts, with palpably established tumors of at least 1000 mm^3, were randomized into two cohorts, experimental and control. The experimental groups received daily o.g. of 200 mg/kg R-etodolac and were sacrificed 24 hr ($n = 3$), 48 hr ($n = 3$), and 72 hr ($n = 3$) postinitiation of treatment. The control cohort was sacrificed at the start of the study.

Statistical analysis of the xenograft experiments

Pairwise differences between the tumor volumes of the treatment groups were compared over time using a permutation test. The null hypothesis for this test is that treatment has no differential effect on the tumor volumes over time. The statistic (SS_Dev) used to test the hypothesis was the sum of the squared differences between the mean tumor volumes summed over all time points. The statistic reflects the amount by which the trajectories of average tumor volume of the two treatment groups are different (Agus et al., 1999b).

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from prostate tumors or the LNCaP cell line using the Trizol reagent (Invitrogen, San Diego, CA). Samples were heated at 95°C for 3 min and snap-cooled before proceeding with DNase I treatment to prevent RNA/DNA hybridization. DNase I (Ambion, Austin, TX) was used to remove any genomic DNA that might interfere with the reaction. Samples were treated with DNase I for 1 hr at 37°C. The RNA yield was quantified spectrophotometrically. Total RNA was reverse-transcribed into cDNA, and PCR was performed in the same reaction using TAQman One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA). The sequences of the primer/probe sets used for this analysis are as follows. F and R are the forward and reverse primers, respectively, and P is the fluorescent-labeled probe. PPARγ: (F) 5′-CGGGCCCTGGCAAAAC-3′, (R) 5′-CGCCCTCGCCTTTGCT-3′, (P) 5′-TTTGTATGACTCATACATAAAGTCCTTCCCGCTG; PPARγ$_2$: (F) 5′-CCCAGAAAGCGATTCCTTCA-3′, (R) 5′-AATGGCATCTCTGTGTCAACCA-3′, (P) 5′-TGATACACTGTCTGCAAACATATCACAAGAAATGACC-3′; β-actin: (F) 5′-GCGCGGCTACAGCTTCA-3′, (R) 5′-TCTCCTTAATGTCACGCACGAT-3′, (P) 5′-CACCACGGCCGAGCGGGA-3′. The mRNA expression of cyclin D1 (Takayasu et al., 2001) and the HER-kinase receptors (Agus et al., 2002) were analyzed using primer sets as previously described.

The one-step RT-PCR cycling conditions for all primer sets were as follows: 30 min at 48°C for RT step; 10 min at 95°C for AmpliTaq Gold activation; and 40 cycles for cDNA dentauring (95°C, 15 s) and annealing/elongation (60°C for 1 min) steps. PCR reactions for each template were done in triplicate using 1 μg of total RNA per sample. Each gene-specific primer pair was tested on standard 384-well plates. Standard curves were constructed using 10–1000 ng of total RNA prepared from the CWRSA6 tumor line. All experiments were optimized such that the threshold cycle (C_T) from triplicate reactions did not differ by more than one cycle number.

The comparative C_T method (PE Applied Biosystems, Foster City, CA) was used to determine relative quantification of gene expression for each gene compared with the β-actin control. First, the C_T values from the β-actin reactions were averaged for each triplicate. Next, the C_T values from the gene-of-interest reactions were averaged. The gene-of-interest average was divided by the β-actin average to take into account the variability of total RNA.

Western blot analysis

Immunoblots were performed as previously described (Towbin et al., 1979) using aliquots of tumor lysates (100 μg per lane) or lysates from LNCaP cells (100 μg per lane). Protein concentration was determined with a Bradford Assay (Bio-Rad, Hercules, CA). The lysates were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose filters. The membranes were incubated overnight at 4°C with antibodies against PPARγ (Sc-7273; Santa Cruz Inc., Santa Cruz, CA), ERK1 (Sc-94; Santa Cruz), phospho-p44/42 MAP kinase (9101S; Cell Signal, Beverly, MA), and cyclin D1 (554180; BD Pharmingen, San Diego, CA), in parallel with anti-β-actin antibodies (A2066; Sigma, St. Louis, MO) and washed well before incubating with the appropriate α-mouse or α-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences, UK).

Cell culture and transfection

Raw 267.4 cells were maintained in Dulbecco's modified eagle medium (DMEM) with high glucose (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. For transfection, cells were seeded in 12-well plates for at least 24 hr prior to transfection. At approximately 50% confluence, cells were transiently transfected using the FuGene transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, with 0.5 μg reporter plasmid $(AOx)_3$-TK-Luc, 0.1 to 0.2 μg of control plasmid pCMXβgal, 0.1 μg PPARγ expression plasmid, and carrier DNA for a total of 1 μg DNA per well. After 16 hr, the cells were washed, and fresh medium containing the appropriate amount of drugs, prepared in 0.5% DMSO, was added to the cells for another 24 hr. The cells were treated with either vehicle alone (DMSO), indomethacin, rosiglitazone, or varying concentrations of R- or S-etodolac.

For luciferase assays, the cells were lysed in potassium phosphate buffer containing 1% Triton X-100, and light emission was detected in the presence of luciferin using a microtiter plate luminometer (MicroBeta TriLux, Gaithersburg, MD). Luciferase values were normalized for variations in transfection efficiency using β-galactosidase as internal control. The results are expressed as relative luciferase units (RLU). The luciferase activity values represent the mean of a minimum of three independent transfections performed in triplicate.

22Rv1 and LNCaP prostate carcinoma cell lines and human monocytic THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin (GIBCO). To assess MAP kinase phosphorylation, 22Rv1 cells were seeded into 100 mm dishes and allowed to attach for a period of 18 hr. The media was then replaced with phenol red-free and serum-free RPMI1640, with 100 μg/ml penicillin and 100 μg/ml streptomycin ± R-etodolac for the indicated times. MAP kinase activation was assessed by Western blot analysis using methods described above. LNCaP cells were treated with R-etodolac at 200, 400, and 600 μM concentrations for 18 hr. Total protein or RNA was isolated

using methods as described previously. Cyclin D1 protein and mRNA expression were assessed by Western blot and quantitative RT-PCR-based assay as previously described.

Flow cytometric analysis of CD36

Human monocytic THP-1 cells were treated for 5 days with 200 μM etodolac (racemic mixture) and 20 μM troglitazone in the presence or absence of 40 ng/ml phorbol ester (TPA) for 48 hr. Cells were fixed, stained with unconjugated anti-CD36 and FITC-conjugated anti-IgG, and analyzed by flow cytometry. A population of 10,000 viable cells was analyzed for each treatment. Data are presented as the difference in mean fluorescence between anti-CD36 antibody and control isotype-matched antibody.

Oil red O staining for neutral lipid uptake

NIH3T3 cells stably expressing recombinant PPARγ (obtained from Dr. Ronald Evans, Salk Institute) were maintained in DMEM supplemented with 10% bovine calf serum (BCS), 100 μg/ml penicillin, and 100 μg/ml streptomycin (GIBCO). The cells were treated for 7 days with the indicated compounds and concentrations and stained for neutral lipids with oil red O as described (Green and Kehinde, 1974).

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