Inhibitors of the Arachidonic Acid Pathway and Peroxisome Proliferator–Activated Receptor Ligands Have Superadditive Effects on Lung Cancer Growth Inhibition

Ingalill Avis, Alfredo Martínez, Jordi Tauler, Enrique Zudaire, Anatoly Mayburd, Raed Abu-Ghazaleh, Frank Ondrey, and James L. Mulshine

Abstract

Arachidonic acid (AA) metabolizing enzymes and peroxisome proliferator–activated receptors (PPARs) have been shown to regulate the growth of epithelial cells. We have previously reported that exposure to the 5-lipoxygenase activating protein–directed inhibitor MK886 but not the cyclooxygenase inhibitor, indomethacin, reduced growth, increased apoptosis, and up-regulated PPARα and γ expression in breast cancer cell lines. In the present study, we explore approaches to maximizing the proapoptotic effects of PPARγ on lung cancer cell lines. Non–small-cell cancer cancer cell line A549 revealed dose-dependent PPARγ reporter activity after treatment with MK886. The addition of indomethacin in combination with MK886 further increases reporter activity. We also show increased growth inhibition and up-regulation of apoptosis after exposure to MK886 alone, or in combination with indomethacin and the PPAR ligand, 15-deoxy-Δ12,14-prostaglandin J2 compared with single drug exposures on the adenocarcinoma cell line A549 and small-cell cancer cell lines H345, N417, and H510. Real-time PCR analyses showed increased PPAR mRNA and retinoid X receptor (RXR)α mRNA expression after exposure to MK886 and indomethacin in a time-dependent fashion. The results suggest that the principal proapoptotic effect of these drugs may be mediated through the known antiproliferative effects of the PPARγ–RXR interaction. We therefore explored a three-drug approach to attempt to maximize this effect. The combination of low-dose MK886, ciglitazone, and 13-cis-retinoic acid interacted at least in a superadditive fashion to inhibit the growth of lung cancer cell lines A549 and H1299, suggesting that targeting PPARγ and AA action is a promising approach to lung cancer growth with a favorable therapeutic index. (Cancer Res 2005; 65(10): 4181-90)

Introduction

Lung cancer is the world’s leading cause of cancer death (1) with conventional therapy still providing only limited success (2, 3). Therefore, translational research to improve outcomes with this disease is critical (4). A number of molecular pathways activated in chronic inflammation may contribute to carcinogenesis (5) and inflammatory mediators may therefore have a role in enabling cancer progression (6, 7). Inflammation is a complex process involving interactions between immune and epithelial cells linking a variety of mediators including eicosanoids (8). We previously reported that tumor growth and tumor cell survival may be neutralized by blocking the 5-lipoxygenase pathway of arachidonic acid (AA) metabolism in lung and breast cancer (9, 10) and that the enzymatic machinery for generating AA metabolites and related intermediaries is variably but frequently expressed in most epithelial cancers (7, 9). We reported that exposure to the 5-lipoxygenase activating protein (FLAP)–directed inhibitor, MK886, but not the cyclooxygenase (COX) inhibitor, indomethacin, reduced growth and increased apoptosis in breast cancer cell lines (10). The exposure of breast cancer cells to MK886 was associated with increased expression of peroxisome proliferator activated receptor (PPAR) α and γ and the same cells were growth inhibited when exposed to relevant PPAR agonists.

Other groups have suggested that MK886 mediates its apoptotic effects through PPARα induction (11, 12). A recent article by Ou et al. suggests that MK886 exerts its apoptotic action through mitochondria (13). MK886 is a potent anticancer drug in vitro and in vivo, but initial clinical studies as a single agent in asthma were disappointing (14).

PPARs are transcription factors that aside from serving as peroxisome proliferator activators also have a central role in lipid metabolism and homeostasis and function by forming heterodimers with the retinoid X receptor (RXRα; refs. 15, 16). The three PPAR subtypes (α, β, and γ) bind to particular fatty acids and their metabolites and regulate the expression of genes involved in the transport and metabolism of these lipidic metabolites within the cell. In mammals, PPARα is highly expressed in brown adipose tissue, followed by liver, kidney, and heart. A recent study suggests that activation of rat liver PPARα provides an antiapoptosis mechanism (17). PPARγ has been shown to be expressed in all tissues studied to date (18, 19). PPARγ is most highly expressed in adipose tissue but also in muscle, colon, and liver. Activation of PPARγ through a synthetic ligand, troglitazone, and other PPARγ activators causes inhibition of proliferation and apoptosis, both in vitro and in vivo, concomitant with a reduction of bcl-2 protein expression (20). The importance of PPAR activation preventing to epithelial carcinogenesis is evident in other tumor systems (21–23). In lung cancer, the PPAR ligand, ciglitazone, has been reported to up-regulate PPARγ mRNA and protein while effecting significant apoptotic and differentiation changes (24). PPARs can mediate inflammation in conjunction with elements from both AA metabolic pathways. These include leukotriene B4 (LTB4), derived from the lipoygenase pathway, and 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2), related to the COX pathway. PGJ2 is a PPARα activator ligand that has been shown to counteract the effects of the
proinflammatory cytokines tumor necrosis factor (TNFα), IL-1, and IL-6 (25). Recently, PGJ₂ has been shown to induce apoptosis involving oxidative stress (26).

In this article, we provide evidence regarding the interaction of the AA and PPAR pathways. This interaction provides an opportunity to develop drug combinations that maximize growth arrest and apoptosis in lung cancer (24). In addition, by using tailored drug combinations, clinical side effects may be reduced because the systemically administered dose of the drug combination is minimized. In this study, we explore drug combinations at low doses in an effort to design new therapies that may improve therapeutic index, especially in early lung cancer.

Materials and Methods

Reagents. The FLAP inhibitor MK886 and PPAR ligands PGJ₂, ciglitazone, LY 171883, and WY-14643 were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). The COX inhibitor indomethacin and the retinoid 13-cis-retinoic acid (13-cis-RA) were purchased from Sigma Chemicals (St. Louis, MO). Polyclonal antibodies to PPARα, β, and γ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell lines. Cell lines used in the study were obtained from the American Type Culture Collection (Manassas, VA). They included small-cell lung cancer cell lines (SCLC) NCI-H355, NCI-N417, and NCI-H510 and non–small cell lung cancer (NSCLC) cell lines A549 and H299. The cells were maintained in RPMI 1640, supplemented with 5% fetal bovine serum (FBS), in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. A transformed human bronchial epithelial cell line, BEAS2B (27), a kind gift from Dr. M. McMenamin (Laboratory of Human Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD), was maintained in serum-free bronchial epithelial medium (BEBM, Cambrex Bio Science Inc., Walkersville, MD) and supplemented following the manufacturer’s recommendations.

Transient transfection and luciferase assay with peroxisome proliferator–activated receptor response element reporter element. Cells were transiently transfected with a thymidine kinase luciferase containing reporter plasmid with a PPAR response element (PPRE), TK-PPREx3-Luc [PPRE × 3 (5′-GTCGACAGGGGACAGGACAAAGG-TCACGTCCGG-GAGTCCGAC), three copies], a kind gift from Dr. Ronald Evans (The Salk Institute, San Diego, CA). Cells were grown in 12-well plates at 60% confluency and cotransfected with 0.5 μg/well of TK-PPREx3-Luc construct and 0.4 μg/well of β-Gal overnight using Lipofectin transfection reagent (Invitrogen). Different treatments were applied in serum-free medium for 24 hours. Then, relative luciferase activity was assayed with a Dual-Light (Invitrogen). Different treatments were applied in serum-free medium for 24 hours. Then, relative luciferase activity was assayed with a Dual-Light (Invitrogen).

To measure apoptosis, we first did immunocytochemical analysis on cytospins from untreated cells or cells treated with indomethacin to ensure induction of PPARγ, then the drugs were removed and PGJ₂ added for an additional period of 24 hours before termination of the assay.

Evaluation of drug interactions in binary and triple combinations. As described in a number of previously published works (28–30), the isobologram method compares the efficacy of the actual drug combination to the efficacy of a theoretical additive model, assuming that the drugs used at the same concentrations as in the actual mix do not interact. The single-drug effects were determined by running a killing curve experiment for all drugs under study in a broader range (IC10-IC90) covering the IC₅₀ range (by additive model) chosen for the drug interaction study.

The nonlinear profiles in the single drug exposure experiments were linearized by using the following function:

\[ F(C) = \ln(\text{survived}) - \ln(\text{killed}) \]

where C is drug concentration.

The linearized single exposure data were fitted to a nonlinear regression F(C) versus C, enabling precise extrapolation between the experimental points of the single exposure experiment. The competing regression functions were assessed and the one that provides the best fit with the experimental data was chosen. In the checkerboard test, incremental dilutions of two anticancer agents were mixed together in 96-well microtiter plates so that each column (eight wells) contained the same composition. The conditions for the binary drug exposure experiment were chosen to maintain IC₅₀ (by additive model) in all points of the isobologram. That means the combined amount of two drugs was chosen for each point to comply with this condition (to be isobolic), based on earlier derived nonlinear extrapolations. Once the amount of the first drug was increased, the amount of the second drug was decreased to provide the fixed theoretical killing efficacy. The actual experimental data were normalized by the computed additive model at each point and these normalized data were plotted in Cartesian coordinates as described in detail in refs. 28–30. The theoretical additive model yields a straight line that crosses the axes X and Y in the points with the coordinates (1, 0) and (0, 1), respectively. The deviation from the straight line indicates drug interaction. The position of the experimental points above the diagonal indicates antagonism and the position below the diagonal indicates synergy. The numerical value of synergy or antagonism can be estimated from the plot by measuring the fractional inhibition coefficient (FIC) at the chosen experimental point. The FIC is computed as the sum of the abscissa and ordinate for the experimental point. The FIC for the straight line depicting the additive model is always 1. The FIC for the concave plots is <1 (the less FIC, the more synergistic is the interaction), and the FIC for the convex plots is grater than 1.

To evaluate the triple combination, the fractional inhibitory concentration index FICI (31) was computed by the equation:

\[ FICI = \frac{D_{A}(ABC)/D_{A}(alone) + D_{B}(ABC)/D_{B}(alone) + D_{C}(ABC)/D_{C}(alone)}{3} \]

where D₅₀(ABC) is the dosage of the component A in the triple combination ABC that yields the desired therapeutic effect, D₅₀(alone) is the dosage of the component A applied individually to reach the same effect. The same applies to the rest of the components. According to the existing conventions, FICI above 1 and below 4 indicates indifference, FICI > 4 indicates antagonism, 0.5 < FICI < 1.0 is defined as superadditivity, whereas FICI < 0.5 is indicative of synergy (32).

Apoptosis analyses. To measure apoptosis, we first did immunocytochemical analysis on cytospins from untreated cells or cells treated with inhibitors utilizing a mouse monoclonal antibody M30 (Roche Molecular Biochemicals). In this assay, the antibody recognizes an epitope of cytokeratin 18 that becomes exposed only after caspase degradation. Detection of the antibody was done using a Histostain Plus kit with AEC substrate (Zymed Laboratories Inc., San Francisco, CA). To cross-validate...
these findings, we used the Apo-One Homogeneous Caspase 3/7 assay (Promega Corporation). These assays were done in 96-well black plates with clear bottoms (Costar 3603, Corning). The drug combination assays were done as described for the proliferation experiments above.

**Reverse transcription and quantitative-real time PCR.** To investigate the levels of PPAR mRNA, real-time PCR was done. After treatment with the indicated drug combinations and time points, cells were washed with PBS and total RNA was extracted using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Total RNA (4 μg) from treated cell samples was retrotranscribed in a final volume of 25 μL using SuperScript First-Strand Synthesis system (Invitrogen) following the manufacturer’s instructions. The quantitative real-time PCR reaction was run in an Opticon cycler (MJ Research, Waltham, MA) using SybrGreen PCR master mix, (Applied Biosystems, Foster City, CA) following manufacturer’s instructions. Thermocycling was done in a final volume of 25 μL containing 2 μL of cDNA (1:10 dilution) and 400 nmol/L of primers: PPARα forward 5′-ACA ACG CGA TTC GTG TTG GA-3′, reverse 5′-GCC CAG AGA TTT GAG ATC TGG A-3′; PPARβ forward 5′-GAG TAC GAG AAG TGT GTA TCT GCA-3′; PPARγ forward 5′-AGC CTC ATG AAG AGC CTT CCA-3′, reverse 5′-GCC CAG AGA TTT GAG ATC TGG A-3′; PPARδ forward 5′-GAG TAC GAG AAG TGT GTA TCT GCA TCC TTC ACA ACG-3′; RXRa forward 5′-GGA AGG TTC GCT AAG CTC TTG C-3′, reverse 5′-TAA GTC ATT TGG TGC GGC G-3′; hypoxanthine phosphor- lytransferase (HPRT) forward 5′-AGC CTC ATG AAG AGC CTT CCA-3′, and HPRT reverse: 5′-ACC CTT GCA TCC TTC ACA ACG-3′. All samples were amplified in triplicates using the following cycle scheme: after initial denaturation of the samples at 95°C for 2 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds were done, fluorescence was measured in every cycle and mRNA levels were normalized by the HPRT values in all samples. A melting curve was run after PCR by increasing temperature from 50°C to 96°C (0.5°C increments). A single peak was obtained for targets, supporting the specificity of the reaction.

**Immunocytochemical analyses.** Tumor cell lines were seeded in six-well tissue culture plates and incubated with various inhibitors for the indicated time points. Cells were harvested and cytosprins prepared. The cells were fixed in 4% formalin and stained with polyclonal antibodies to PPARs using the Histostain plus kit as above.

**Statistical evaluation.** Statistical significance of differences between samples (with a minimum of six wells per sample) was determined using unpaired Student’s t test in Excel. P < 0.05 was regarded as statistically significant.

**Results**

**Combined drug effects increase peroxisome proliferator-activated receptor γ reporter activity and enhance growth reduction.** To further explore the relevance of the observation on PPAR up-regulation after MK886 drug exposure as previously
published (10), we used a PPARγ reporter construct in the lung cancer cell line, A549. We did two separate experiments evaluating exposure to 0.5 to 2.0 μmol/L MK886. PPARγ reporter activity was significantly increased after exposure to 1 or 2 μmol/L MK886 in a dose-dependent manner and is expressed in RLU (Fig. 1A). As observed in Fig. 1B, the exposure of lung cancer cells to either 2 μmol/L MK886 or 10 μmol/L indomethacin increased reporter activity. When the two drugs were combined, a higher reporter activity was observed when compared with treatment with individual drugs.

From previous work, we knew that exposure of breast cancer cell lines to indomethacin alone results in minimal growth inhibitory effects (10). To further study the biological effect of combining AA inhibitors with PPAR ligands on lung cancer cell proliferation, we began by analyzing individual and combined effect of several of these compounds. As summarized in Table 1, we evaluated both SCLC and NSCLC cell lines to determine the broader range of biological effect of AA inhibitors and PPAR ligands as shown by the MTT assay. MK886 inhibited growth of all lung cancer cell lines evaluated. Indomethacin had minimal effect on growth even at high concentrations (>10 μmol/L). We then investigated the potential benefits in terms of enhanced cell growth reduction provided by the combination of the two antagonists, MK886 and indomethacin. A combination of 0.5 μmol/L MK886 and 10 μmol/L indomethacin was more efficient in reducing growth than MK886 alone on cell lines A549 and N417 (P < 0.05), but not on cell line H345. Of the PPAR ligands, only PGJ2 exposure elicited a consistently potent growth inhibition for the cell lines tested. Another PPARγ agonist, ciglitazone, was associated with a significant growth inhibitory effect for cell line A549 and N417 but not for H345 (Table 1). We were particularly interested in drug effects on A549 because this is an adenocarcinoma that is the dominant form of lung cancer seen today clinically. Figure 1C shows representative growth curves for cell line A549 exposed to increasing doses of MK886 and PGJ2, as measured by the MTT assay. Both drugs inhibited growth in a dose-dependent manner, with maximum growth inhibition occurring at 4 μmol/L (P < 0.05).

### Table 1. Maximal effect of AA inhibitors and PPAR ligands on lung cancer cell line growth

<table>
<thead>
<tr>
<th>Cell line</th>
<th>A549</th>
<th>H345</th>
<th>N417</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA inhibitor</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MK886, 0.5 μmol/L</td>
<td>50 ± 6</td>
<td>25 ± 5*</td>
<td>47 ± 8*</td>
</tr>
<tr>
<td>Indomethacin, 10 μmol/L</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MK + indomethacin, 0.5 μmol/L + 10 μmol/L</td>
<td>75 ± 10*</td>
<td>27 ± 8*</td>
<td>60 ± 5*</td>
</tr>
<tr>
<td>PPAR ligands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WY 14643 (α), 4 μmol/L</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LY 171883 (γ), 4 μmol/L</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ciglitazone (γ), 4 μmol/L</td>
<td>36 ± 12*</td>
<td>NS</td>
<td>35 ± 5*</td>
</tr>
<tr>
<td>PGJ2 (γ), 4 μmol/L</td>
<td>50 ± 10*</td>
<td>90 ± 10*</td>
<td>85 ± 5*</td>
</tr>
</tbody>
</table>

Note: Cell lines A549, H345, and N417 were treated with AA inhibitors or PPAR ligands for 3 days and growth inhibition was analyzed using an in vitro proliferation assay. Results are expressed as mean percent growth inhibition ± SD of three different experiments. Abbreviation: NS, nonsignificant. *P < 0.05, significantly different from control.
To further analyze the potential interactions between the AA metabolic pathways and the PPAR activation observed in the PPARγ reporter assay, we did sequential treatments. The AA inhibitors were added first for 24 hours because we have previously shown that this induced expression of PPARγ, and then we added the PPARγ ligand, PGJ2. In these combination experiments, we used MK886 and PGJ2 drug concentrations that were less than optimally inhibitory to be able to observe drug combination effects more readily. NSCLC cell line A549 was treated with 0.5 μmol/L MK886, 10 μmol/L indomethacin, and a combination of the inhibitors for 24 hours. When a low concentration of 0.5 μmol/L PGJ2 was applied for another 24 hours (a dose of PGJ2 that by itself has minimal growth inhibitory effect using the MTT assay), growth was reduced when compared with either drug alone (P < 0.05; Fig. 1D). This growth inhibition was confirmed by a decrease in DNA synthesis (Fig. 1E), and these results are consistent with a PPARγ-mediated growth effect.

**Exposure to inhibitor combinations induces apoptosis through caspase activation.** We next evaluated whether an apoptotic pathway was involved in the growth inhibitory response to the inhibitors and ligands. Using an immunocytochemical assay with an antibody (M30) for the detection of a caspase cleavage product of cytokeratin 18, we evaluated the effects of MK886, indomethacin, and PGJ2 in this early event of the caspase cascade. The results of these experiments are shown for cell line A549 (Fig. 2A). MK886 induced 25% apoptosis at a concentration of 0.5 μmol/L, whereas neither indomethacin nor PGJ2 had a significant effect by themselves. When the drugs were combined, we observed an increase in apoptotic cells by 50% with the combination of MK886, indomethacin, and PGJ2.

The effect of the drugs on caspase 3 and 7 activation was also evaluated. As shown in Fig. 2B, exposure of A549 cells to low doses of MK886 or indomethacin alone showed no significant increase in apoptosis when compared with untreated cells. However, when the two drugs were combined, we observed an increase in apoptosis. Apoptosis was further increased by 50% in the presence of 0.5 μmol/L PGJ2. We next tested the drug combinations on SCLC cells. When H345 cells were treated with 0.5 μmol/L MK886, a significant increase in apoptosis was observed (Fig. 2C) compared with untreated cells, but indomethacin had no significant apoptotic induction effect on H345. In contrast to the other cell lines when 0.5 μmol/L MK886 was combined with 10 μmol/L indomethacin, there was less apoptotic activity compared with MK886 alone. In the presence of 0.5 μmol/L PGJ2, no additional significant increase in caspase activity could be determined compared with MK886 alone (Fig. 2C). Comparable results were observed with SCLC cell line H510 (Fig. 2D). These results are consistent with the growth inhibition observations shown above (Table 1; Fig. 1D and E). The differential response with NSCLC cell lines responding more favorably than SCLC cell lines is of mechanistic interest.

**MK886 regulates peroxisome proliferator–activated receptor γ and retinoid X receptor α mRNA expression levels over time.** The proapoptotic effect of PPARγ is thought to require binding to retinoic acid receptor to form a heteroduplex that is transcriptionally active. A549 cells were cultured in the presence of 1.0 μmol/L MK886 for 6, 12, and 24 hours and analyzed by real-time PCR for subsequent changes in the PPARγ and RXRα mRNA expression levels relative to an untreated control (Fig. 3A). We observed a 1.4-fold increase in PPARγ after 6 hours treatment with MK886, with a maximum of 1.8-fold increase at 12 hours. PPARγ mRNA levels declined below baseline levels by 24 hours. A similar pattern was observed with PPARα (data not shown). A maximum up-regulation of RXRα mRNA was observed as early as 6 hours after treatment with MK886.

**Figure 3.** A, effect of MK886 on PPARγ and RXRα mRNA expression levels over time, by real-time PCR. Analyses in cell line A549 after 6, 12, and 24 hours exposure to 1 μmol/L MK886. Open columns, RXRα; filled columns, PPARγ. Results are expressed as fold increase of untreated control cells with no drug exposure. B, effect of MK886 and/or indomethacin on PPARγ and RXRα mRNA expression levels. A549 cells were exposed for 12 hours to 0.5, 1.0, and 2.0 μmol/L MK886, 10 μmol/L indomethacin, and a combination of the two drugs. Open columns, PPARγ; filled columns, RXRα. Results are expressed as relative mRNA after normalization to HPRT. C, effect of MK886 and/or indomethacin mRNA expression levels of PPARα, γ, α, and δ. A549 cells were treated with 1.0 μmol/L MK886, 10 μmol/L indomethacin, and a combination of the two drugs. Open columns, PPARα; filled columns, PPARγ; striped columns, PPARα. Results are expressed as relative mRNA after normalization to HPRT.
as 6 hours. Induction was still elevated at 12 hours, then returned to baseline levels by 24 hours.

Retinoid X receptor α and peroxisome proliferator–activated receptor α, β, and γ mRNA expression levels after exposure to MK886 and/or indomethacin. We then looked at the mRNA expression pattern after combination of MK886 and indomethacin (Fig. 3B). These results show that MK886 augmented RXRα and PPARγ expression in a dose-dependent fashion. In contrast, indomethacin under these conditions had no effect on the expression of either gene. A combination of 2.0 μmol/L MK886 and indomethacin up-regulated RXRα, but not PPARγ (Fig. 3B). In other experiments, we evaluated the effect of MK886 and indomethacin on all the isoforms of PPAR (α, β, and γ). As shown in Fig. 3C, MK886 increased PPAR α, β, and γ, but the addition of indomethacin had no additional effect on PPARγ expression, although it slightly elevated α and β. A combination of the two drugs showed a slight increase in expression of all PPAR mRNAs.

Treatment with inhibitors on lung cancer cell lines causes nuclear translocation of peroxisome proliferator–activated receptor γ. In light of the mRNA induction results, we wanted to determine if there is a difference in the protein distribution after treatment with the inhibitors. We did immunocytochemical experiments using polyclonal antibodies against the three different PPARs on two cell lines, NSCLC A549 and SCLC H345 (Table 2). We observed in A549 that treatment with MK886, indomethacin, or a combination of both drugs for 12 hours, induced a translocation of the PPARγ protein from the cytoplasm to the nucleus, whereas PGJ2 did not change its distribution. The protein distribution of PPARs in untreated cells seems to be cell line dependent, being mainly cytoplasmic in NSCLC cell line A549 and nuclear in SCLC cell line H345. The levels of protein expression were consistent with the results of the mRNA expression experiment with the drug combination of MK886 and indomethacin resulting in elevated PPARγ immunoreactivity in the cell line, A549. To confirm these scenarios...

Table 2. Analyses of PPAR protein distribution in lung cancer cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Antibody</th>
<th>Staining index (intensity/distribution)</th>
<th>0.5 μmol/L MK</th>
<th>10 μmol/L Indo</th>
<th>MK + Indo</th>
<th>0.5 μmol/L PGJ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>PPARα</td>
<td>2/1 CY</td>
<td>Blush CY</td>
<td>3/3 NU</td>
<td>2/2 CY/NU</td>
<td>2/2 CY/NU</td>
</tr>
<tr>
<td></td>
<td>PPARγ</td>
<td>2/2 CY</td>
<td>4/3 CY/NU</td>
<td>4/3 CY/NU</td>
<td>2/3 CY/NU</td>
<td>1/1 CY</td>
</tr>
<tr>
<td></td>
<td>PPARβ</td>
<td>3/4 CY</td>
<td>1/2 CY</td>
<td>2/2 CY</td>
<td>3/3 CY</td>
<td>1/2 CY/NU</td>
</tr>
<tr>
<td>H345</td>
<td>PPARα</td>
<td>1/2 NU</td>
<td>Blush NU</td>
<td>1/2 NU</td>
<td>1/1 NU</td>
<td>1/1 CY</td>
</tr>
<tr>
<td></td>
<td>PPARγ</td>
<td>1/2 NU</td>
<td>2/2 NU</td>
<td>Negative</td>
<td>1/2 NU/CY</td>
<td>3/3 NU/CY</td>
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<tr>
<td></td>
<td>PPARβ</td>
<td>1/2 NU/CY</td>
<td>Negative</td>
<td>1/2 CY</td>
<td>3/2 CY</td>
<td>Negative</td>
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</table>

NOTE: Cell lines A549 and H345 were treated for 12 hours with 0.5 μmol/L MK886, 10 μmol/L indomethacin, and 0.5 μmol/L PGJ2. Cytospins were prepared and fixed in 4% formalin. Immunocytochemical analyses were done using polyclonal antibodies to PPARα, γ, and β using a Zymed Histostain kit and scored as previously described (10).

Abbreviations: MK, MK886; Indo, indomethacin; CY, cytoplasmic; NU, nuclear.
findings, we did earlier time point studies (Fig. 4), and again observed that PPARγ immunoreactivity is localized in the cytoplasm at baseline but moves into the nucleus in some cells at the lower MK886 dose at 1 hour. The timing of the first morphologic evidence of apoptosis after MK886 exposure was quite rapid; by 2 hours there is both strong nuclear expression of PPARγ as well as overt evidence of nuclear condensation and blebbing in the A549 cells (Fig. 4). This observed translocation may be additional evidence of PPAR activation and induction of apoptosis because the recruitment of PPAR to the nucleus is needed for its downstream effects.

**Low-dose drug combinations with ciglitazone, MK886, and 13-cis-retinoic acid reduces lung cancer growth.** We have preliminary experimental evidence indicating MK886 exposure causes a rapid apoptotic response as well as a more sustained apoptotic response over 24 hours, suggesting that at least two possible mechanisms of cell death occur after MK886 exposure. The preponderance of our experimental data suggests that MK886 apoptotic induction is at least in part mediated through an induction of PPARγ. Because RXRa is the known transcriptional partner of PPARγ, we evaluated whether the addition of a retinoid may further enhance the apoptotic/growth inhibitory effect. An earlier report suggested that PPARγ transcription could be enhanced by a PPAR agonist in non–small cell lung cancer (24). For these experiments, we evaluated a panel of PPARγ ligands, and ciglitazone was the most potent.

The drug combinations with MK886, ciglitazone, and 13-cis-RA was evaluated in A549 cells and BEAS2b cells. The cell line BEAS2b is a transformed bronchial epithelial cell line that does not exhibit fully invasive phenotype and was used as a surrogate of evolving cancer. As shown in Fig. 5A and C, a profound growth inhibitory effect was observed with the three drug combinations on both cell lines with drug combinations of 0.5 μmol/L MK886, 1.0 μmol/L ciglitazone, and 1.0 μmol/L 13-cis-RA for both cell lines. To reduce potential adverse drug effects, the drug concentrations were all reduced (Fig. 5B and D), and under these conditions, the combined drugs still resulted in superadditive growth inhibition. To confirm that these observations were not unique for cell line A549, we explored these low-drug combinations on another NSCLC cell line, H1299 with similar results (Fig. 5E).

**Figure 5.** Low drug combinations with 13-cis-RA reduce growth. Cell lines A549 (A, B) or BEAS2B (C, D) and NSCLC cell line H1299 (E), were incubated for 48 hours with low concentrations of MK886, ciglitazone (Cig), and 13-cis-RA (RA), and growth assessed by the MTT proliferation assay. Results are expressed as percent growth of control. Experiments were repeated thrice. *, P < 0.05 (significantly different from control); ^, P < 0.05 (significantly different from single drug).
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Table 3. Formal analysis of binary and triple drug combinations

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Survival (%) A549</th>
<th>FICI</th>
<th>Survival (%) H1299</th>
<th>FICI</th>
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<tbody>
<tr>
<td>MK886</td>
<td>13-cis RA</td>
<td>Cigliotzone</td>
<td>0.1</td>
<td>0</td>
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<tr>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>42 ± 5*</td>
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<td>1.6</td>
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<td>0</td>
<td>0.25</td>
<td>0</td>
<td>89 ± 12</td>
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NOTE: Cell lines A549 and H1299 were treated with binary or triple combinations of MK886, ciglitazone, and 13-cis RA and evaluated by the MTT proliferation assay. Fractional inhibitory concentration index was computed for single and combined drug treatments. The drug concentrations are in micromoles.

*aP < 0.05, significantly different from control.

Formal analysis of binary and triple drug interactions. In Table 3, we show single, double, and triple drug exposures that are required to conduct formal analysis for drug interactions using FICI methodology as described in Materials and Methods. This FICI analysis indicates that positive interaction exists among all the components of the combinations in most of the conditions, and the extent of this interaction can be defined as superadditive as indicated by an index of <1. The most inhibitory result for A549 was seen with the combined exposure to 0.8 μmol/L MK886, 2 μmol/L of 13-cis RA, and 0.25 μmol/L of ciglitazone, in which only 3% of the cells survived the combined exposure which suggests superadditive drug interactions. For the cell line H1299, the FICI analysis suggests that the interaction of 0.1 μmol/L MK886, 0.25 μmol/L of 13-cis RA, and 2 μmol/L of ciglitazone may be synergistic with an index of 0.48. From the analyses of the combination experiments, inclusion of MK886 seems to potentiate the effect of both ciglitazone and 13-cis RA as well as of their combination only when MK886 individual concentration enters its dynamic range of growth suppression effect (Table 3). Independent analysis of the double drug combination, ciglitazone and 13-cis RA, by isobologram also indicated at least superadditivity (28–30).

Discussion

For the range of lung cancer cells tested, including NSCLC and SCLC cell lines, we have seen significant inhibitory effects with MK886 in combination with PPAR ligands at clinically achievable doses. For this study, we used the peripheral airway-derived adenocarcinoma cell line, A549, because adenocarcinoma is currently the most common form of lung cancer. The metabolites of the AA pathway have been implicated in the promotion of cancer (33–35). Although it is evident that AA can be metabolized either by the 5-lipoxygenase or the COX pathway, the relative importance of each pathway varies with the cell type (36, 37). We have previously reported that the FLAP inhibitor, MK886, has significant effect on leukotriene biosynthesis growth inhibition and apoptosis (9). Inhibitors of 5-lipoxygenase activity may act through several mechanisms, including redox interactions (37). MK886 has also been shown to induce apoptosis independently of both 5-lipoxygenase and FLAP. Furthermore, induction of differentiation and apoptosis in cancer cells can also occur through the action of other oxidation products of AA (11, 38, 39). In this article, we focus on the effect of MK886 on PPAR induction.

The COX inhibitor indomethacin had little in vitro effect on lung cancer cell line growth as we previously reported for breast cancer cell line growth even at high concentrations (>10–40 μmol/L; refs. 7, 40). This contrasts with other findings in the literature, although doses >10 μmol/L were used in those other studies (41, 42). In the present study, we confirmed that indomethacin did not inhibit growth or induce apoptosis when tested on lung tumor cell lines. However, we have previously reported that in vitro results may not predict for in vivo activity (7, 43). Hence, these data do not exclude an important in vivo contribution of COX activity especially in combination with other drugs recruiting PPARγ activity. We have found that MK886 in combination with indomethacin increased PPARγ reporter activity. However, for the purposes of this in vitro study, we investigated the drug combinations that may enhance cancer cell killing. Indomethacin, when coincubated with low concentrations of MK886, did not significantly inhibit growth. These two drugs are structurally similar, both being indole acetic acid derivates (11). The growth inhibition was confirmed by a decrease in DNA synthesis and induction of early apoptosis as measured by caspase activity, indicating that blocking both arms of AA metabolism may have important effects on growth regulation.

Recently, induction of apoptosis in response to PPARα and γ activation has been reported in a variety of normal and neoplastic cells (11, 19). In our previous study with breast cancer cells, a 5-lipoxygenase inhibitor up-regulated PPARα and γ expression, and the growth of these cells was inhibited upon exposure to relevant PPAR agonists (10). To evaluate whether the PPAR up-regulation contributed to the growth inhibition and apoptotic effect induced by the combination of MK886 and indomethacin, we exposed lung cancer cell lines to the inhibitors alone and then to a PPAR ligand. The combined drug exposure resulted in significantly less growth and increased proapoptotic activity than were seen with the individual exposures alone. Therefore, the increased anti-proliferative effects of combination drug exposure may relate to the observed increase in PPARγ message. In A549, both MK886 and indomethacin induced the expression of PPARγ above control. Both PGJ2 and ciglitazone alone, but not LY 171883 or the PPARγ ligand, WY 14643, had potent growth inhibitory effect on all or most of the lung cancer cell lines tested.

Protein expression of the PPARs in the lung cancer cell lines generally correlated with the mRNA studies, with all tested drugs elevating the levels of PPARγ in cell line A549, except for PGJ2. For the final combination experiments, we did not use PGJ2 because this ligand is not available clinically; it has been reported to exhibit complex drug effects (44, 45), and PGJ2 also had minimal effects in our experience with inducing PPAR protein expression. We used ciglitazone for our lead PPAR ligand because it was the most potent of the PPARγ agonists tested and it had.

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the most significant growth inhibitory effect in combination with MK886 in the in vitro assays. We also selected to work with ciglitazone for our subsequent combination experiments rather than indomethacin because ciglitazone was consistently more potent than indomethacin.

For the remainder of our analysis, we focused on strategies to maximize the growth inhibitory effect of PPARγ on NSCLC. We evaluated the combinations of MK886, ciglitazone, plus 13-cis-RA, formally evaluating the nature of the interaction of these drugs. The most potent effect was observed with the combination of MK886, 13-cis-RA, and ciglitazone at doses of 0.8, 2.0, and 0.25 μmol/L, respectively. At these doses, the interaction of these drugs is a superadditive growth inhibitory effect (31).

A superadditivity effect was originally reported with the optimal interaction of RXRα-PPARγ heterodimers (46). In a recent report, the combinations of three inhibitors have been tested, combining PPARγ, and RXRα ligands with the proteasome inhibitor MG132 or MG262 (47). Triple drug combinations caused an increase in the luciferase PPRE activation as compared with the dual combination of PPARγ and RXRα ligands (47). These authors suggest that this accumulation can be attributed to the impaired proteasomal degradation of the heterodimer components. The RXR and PPAR ligand combination results for lung cancer are also consistent with the earlier published results for leukemia and breast cancer, showing enhanced therapeutic benefit (48, 49).

The use of a drug combination approach in reducing drug toxicity is a critical issue in considering drug therapy in the setting of early lung cancer because the doses required for superadditive growth inhibition are modest as previously suggested (24). The recent success with chemotherapy trials suggesting a benefit to adjuvant therapy for early lung cancer heightens the need for identifying effective drug combinations with a favorable therapeutic index for early lung cancer. If this approach is successful in vivo, this combination would also be appealing to try in chemoprevention settings (50, 51). Because drug therapy becomes more tailored to the particular requirement of early lung cancer, the feasibility of locally delivering drug combinations as an integrated aerosol drug construct emerges as a strategy to further optimize therapeutic index (52).

In summary, whereas biochemical inhibitors of AA metabolism and PPAR ligands differ in their ability to inhibit growth and stimulate apoptosis in lung cancer cell lines, combinations of these agents result in at least superadditive inhibition of growth and induction of apoptosis. Further investigation into the mechanistic basis and formal analysis of these drug interactions needs to be conducted for the development of these approaches. Nevertheless, our study provides a framework for considering rationally designed drug combinations based on modulating the proapoptotic activity of PPARγ for lung cancer drug therapy.

Acknowledgments

Received 9/22/2004; revised 2/16/2005; accepted 3/3/2005.

Grant support: Spanish Ministry of Science and Education grant BU2004- 02383/BFI.

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References


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