COLORECTAL CANCER

Synergistic effects of RXR α and PPAR γ ligands to inhibit growth in human colon cancer cells—phosphorylated RXR α is a critical target for colon cancer management

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Background and aims: The activation of the peroxisome proliferator-activated receptor γ (PPAR γ) that forms heterodimers with retinoid X receptors (RXRs) elicits an antineoplastic effect on colorectal cancer. It was previously reported that the accumulation of the non-functional phosphorylated form of RXR α (p-RXR α) interfered with its signalling and promoted the growth of hepatoma cells. In this study the effects of p-RXR α on the ability of RXR α and PPAR γ ligands to inhibit growth in colon cancer cells was examined.

Methods: The effects of the combination of the PPAR γ ligand ciglitazone and the RXR α ligand 9-*cis*-retinoic acid (RA) on inhibition of cell growth in Caco2 human colon cancer cells which express high levels of p-RXR α protein were examined

Results: The RXR α protein was phosphorylated and also accumulated in human colon cancer tissue samples as well as human colon cancer cell lines. When the phosphorylation of RXR α was inhibited by the MEK inhibitor PD98059 or by transfection with a point-mutated RXR α , which mimicked the unphosphorylated form, the combination of 9-*cis*RA and ciglitazone synergistically inhibited the cell growth and induced apoptosis. The combined treatment with these agents also caused a decrease in the expression levels of both cyclo-oxygenase-2 (COX-2) and c-Jun proteins and mRNAs. Reporter assays indicated that this combination induced the transcriptional activity of the peroxisome proliferator-responsive element promoter and also inhibited that of the AP-1 promoter.

Conclusion: A malfunction of RXR α due to phosphorylation is associated with colorectal cancer. Therefore, the inhibition of phosphorylation of RXR α and the activation of the RXR-PPAR γ heterodimer by their respective ligands may be useful in the chemoprevention and/or treatment of colorectal cancer.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear hormone receptor superfamily that has been shown to play an important role in the regulation of lipid homeostasis, energy metabolism, inflammatory responses and the induction of apoptosis.^{1,2} Recent studies have indicated that the aberrant expression of PPAR γ has been observed in several types of human malignancies, including colorectal cancer.^{3–5} Moreover, both *in vivo* and *in vitro* studies have demonstrated that PPAR γ agonists can inhibit cell growth, cause apoptosis and thus exert antitumour effects in human colon cancer.^{5–7} These reports suggest that the activation of PPAR γ by its ligand may be a potentially useful strategy for the chemoprevention and/or treatment of colorectal cancer.

PPARs are ligand-activated transcription factors. As a result, after ligand binding, PPAR γ can regulate gene expression by binding to the peroxisome proliferator-responsive element (PPRE) in target genes as a heterodimer with retinoid X receptors (RXRs).^{8–9} RXR consists of three subtypes, α , β and γ .¹⁰ Among them, we previously found a malfunction of RXR α due to post-translational modification by phosphorylation to be associated with carcinogenesis of the liver.^{11,12} The phosphorylated form of RXR α (p-RXR α) lost its transactivation activity via the RXR-responsive element and interfered with the function of remaining normal RXR α in a dominant-negative manner, thereby promoting the growth of hepatoma cells.¹¹ Exogenous 9-*cis*-retinoic acid (RA), a natural ligand for RXRs, induced the degradation of RXR α while also restoring the function of this receptor in human hepatoma cells.¹¹ These findings may explain how the retinoid can inhibit the growth of hepatoma cells in both clinical^{13,14} and *in vitro* studies,^{15,16} thereby leading

us to hypothesise that the phosphorylation of the RXR α protein may thus play a role in other types of human malignancies, including colorectal cancer.

In view of these observations, there has been considerable interest in utilising the combination of ligands for PPAR and RXR for the prevention and treatment of various types of cancer.^{17–20} In the present study, we examined in detail the effects of such combined treatment on the inhibition of cell growth in Caco2 human colon cancer cells which express high levels of phosphorylated RXR α (p-RXR α) protein. We also transfected Caco2 cells with the mutant RXR α which expressed the unphosphorylated form of RXR α , and we examined the effects of the combination of these ligands on the cyclo-oxygenase-2 (COX-2) expression in these cells, since COX-2 expression has been shown to play a crucial role in the development of human colorectal cancer.^{21,22}

MATERIALS AND METHODS

Materials

9-*cis*RA and PD98059 (a MEK inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ciglitazone (cig)

Abbreviations: cig, ciglitazone; COX, cyclo-oxygenase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; p-RXR α , phosphorylated RXR, retinoid X receptor α ; RA, retinoic acid; RT-PCR, reverse transcription-PCR; RXR, retinoid X receptor; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling

was from Calbiochem (La Jolla, CA, USA). RPMI 1640 medium and fetal calf serum (FCS) were both from Invitrogen (Carlsbad, CA, USA).

Plasmid construction

Histidine-tagged wild-type RXR α was constructed using pRShRXR α (kindly provided by Dr RM Evans, The Salk Institute, La Jolla, CA, USA), by PCR. The point-mutated RXR α T82A/S260A, in which both Thr82 and Ser260 are mutated to alanine, which are putative phosphorylation sites for mitogen-activated protein kinases (MAPKs), and thus mimicking the unphosphorylated form of RXR α protein, has been previously described.¹¹

Cell lines and cell culture

The Caco2, HT29, colo320, colo201, DLD-1 and SW837 human colorectal cancer cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The HCT-116 human colorectal cancer cell line and the FHC normal human fetal colon cell line were obtained from ATCC (Manassas, VA, USA). All of the cell lines were maintained in RF10 medium containing RPMI 1640 medium supplemented with 10% FCS. The cells were cultured in an incubator with humidified air at 37°C with 5% CO₂.

Cell proliferation assays

Four thousand Caco2 cells were seeded into multiple 35 mm diameter dishes in RF10 medium, and 24 h later they were treated with 5 μ M 9-*cis*RA alone, 10 μ M cig alone or a combination of these agents in the presence or absence of 50 μ M PD98059 for 24 h. The doses of these reagents were selected according to our previous¹¹ study and Yang *et al.*²⁰ The numbers of viable cells in replica plates were then counted using the trypan blue dye exclusion method, as previously described.²³

Apoptosis assays

Caco2 cells were treated with 5 μ M 9-*cis*RA alone, 10 μ M cig alone or a combination of these agents in the presence or absence of 50 μ M PD98059 for 24 h. To quantify the induction of apoptosis, a DNA fragmentation assay was performed using the Cell Death Detection ELISA^{PLUS} kit, (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions, as described previously.¹⁶ The TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) method was also performed using the *In situ* Cell Death Detection Kit (Roche Diagnostics), which visualises apoptotic cell death by detecting DNA strand breaks in the individual cells, as described previously.²³

Protein extraction and western blot analysis

Total cellular protein was extracted and equivalent amounts of protein were examined by a western blot analysis, as previously described.¹¹ Polyclonal anti-RXR α (Δ N197) and anti-COX2 (C-20) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal anti-c-Jun (clone 3) antibody was from BD Biosciences (Franklin Lakes, NJ, USA). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon International (Temecula, CA, USA). An antibody against GAPDH was used as a loading control. The intensities of the blots were quantified using the NIH Image software version 1.61.

RNA extraction and semi-quantitative reverse transcription-PCR analysis

Both RNA extraction and a reverse transcription-PCR (RT-PCR) analysis were performed as described previously.²⁴ Total RNA

was isolated from Caco2 cells using ISOGEN (WAKO Pure Chemical, Osaka, Japan). cDNA was amplified from 1 μ g of total RNA using a High Fidelity RNA PCR Kit (TAKARA Biomedicals, Tokyo, Japan), according to the manufacturer's instructions. c-Jun-, COX-2- and GAPDH-specific primer sets and the amplification cycle conditions were as previously described.²⁴⁻²⁶ Using a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA, USA), 25- and 30-cycle rounds of PCR were chosen for an optimum analysis of the expression of c-Jun and COX-2 mRNAs, respectively. The intensities of the mRNA bands were then quantified using the NIH Image software package version 1.61.

Luciferase reporter assays

Reporter assays were performed as described previously.¹¹ The PPRE-luciferase reporter plasmid PPRE₃-TK-LUC was kindly provided by the late Dr K Umehono (Kyoto University, Kyoto, Japan).²⁷ The AP-1 promoter luciferase reporter plasmid pAP-1-Luc was purchased from Stratagene (La Jolla, CA, USA). Caco2 cells were co-transfected with a combination of wild-type or a mutant hRXR α -expressing plasmid (300 ng/35 mm dish) with PPRE₃-TK-LUC or pAP-1-Luc reporter (750 ng/35 mm dish), along with pRL-CMV (*Renilla* luciferase, 100 ng/35 mm dish; Promega, Madison, WI, USA) as an internal standard to normalise the transfection efficiency. Transfections were performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. After exposure of the cells for 24 h to the transfection mixture, the cells were treated with vehicle, 5 μ M 9-*cis*RA, 10 μ M cig or the combination of these agents for 24 h. Thereafter, the cell lysates were prepared and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega). Changes in the firefly luciferase activity were calculated and plotted after normalisation with changes in *Renilla* luciferase activity in the same sample.

Tissue specimens

Colon cancer and its surrounding non-cancerous colon tissues were obtained by surgical resections from eight patients. This study was approved by the Ethics Committee of the Gifu University School of Medicine, and all of the patients gave their written informed consent.

Statistical analysis

The data are expressed as the mean (SD). Statistical significance of the difference in the mean values was assessed with one-way analysis of variance (ANOVA), followed by Sheffe's *t* test.

RESULTS

The RXR α protein is phosphorylated in colon cancer cells

In our initial study we examined whether or not the RXR α protein is constitutively phosphorylated in a series of human colorectal cancer cell lines and the FHC normal human fetal colon cell line (fig 1). The anti-RXR α (Δ N197) antibody, which can be regarded as the specific antibody for the phosphorylated form of RXR α protein, was utilized for the western blot analysis.^{11, 28} We found that the level of the p-RXR α protein was constitutively increased in all seven colorectal cancer cell lines (Caco2, HT29, Colo201, Colo320, DLD-1 HCT-116 and SW837) that we examined in this study. On the other hand, the p-RXR α protein was not detected in the FHC cell line (fig 1, lanes 1). We also found a marked decrease in the level of this protein when the cells were treated with the MEK inhibitor PD98059 alone (fig 1, lanes 3) or the combination of 9-*cis*RA plus PD98059 (fig 1, lanes 4) in all of these colon cancer cells.

Figure 1 Levels of the phosphorylated form of the retinoid X receptor α (RXR α) protein in colon cancer cell lines and the FHC normal human colon epithelial cell line. Seven human colon cancer cell lines Caco2, HT29, Colo201, Colo320, DLD-1, HCT-116 and SW837 cells and FHC normal human colon epithelial cells were treated with vehicle (lane 1), 5 μ M 9-*cis*-retinoic acid (RA) (lane 2), 50 μ M PD98059 (lane 3) or the combination of 50 μ M PD98059 and 5 μ M 9-*cis*RA (lane 4) for 24 h. Total protein extracts were prepared from the indicated cell lines, and equivalent amounts of protein (15 μ g/lane) were then examined by a western blot analysis for the phosphorylated RXR α protein. An antibody against glyceraldehyde-3-phosphate dehydrogenase served as a loading control. The intensities of blots were quantitated by densitometry, and the values are shown as bar graphs in the lower panel. Repeat western blot analyses gave similar results. The asterisks represent a significant difference ($p < 0.01$) in comparison with column 1. Bars show the SD.

Figure 2 Effects of the combination of 9-*cis*-retinoic acid (RA) plus ciglitazone (cig) on the cellular levels of the phosphorylated retinoid X receptor α (p-RXR α) protein in Caco2 cells. The cells were treated with vehicle (lanes 1 and 5), 5 μ M 9-*cis*RA (lanes 2 and 6), 10 μ M cig (lanes 3 and 7) or the combination of 5 μ M 9-*cis*RA plus 10 μ M cig (lanes 4 and 8) for 24 h in the absence (lanes 1–4) or presence (lanes 5–8) of 50 μ M PD98059, and cell extracts were examined by a western blot analysis using an antibody specific to phosphorylated RXR α (Δ N197). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. The intensities of the blots were quantitated by densitometry and the values are shown as a bar graph in the lower panel. Similar results were obtained in a repeat experiment. The asterisks represent a significant difference ($p < 0.01$) in comparison with columns 1 and 5. Bars show the SD.

9-*cis*RA plus cig cause a synergistic decrease in the p-RXR α protein in Caco2 cells in the presence of MEK inhibitor

We next examined whether 9-*cis*RA and the PPAR γ agonist cig inhibit the phosphorylation of RXR α in Caco2 cells, which express both the RXR α and PPAR γ mRNAs.²⁹ There was no remarkable change in the expression level of p-RXR α when the cells were treated with 9-*cis*RA alone, cig alone or the combination of these ligands in the absence of PD98059 (fig 2, lanes 1–4). However, when the cells were treated with these ligands in the presence of PD98059, there was a decrease in the expression level of p-RXR α proteins (fig 2, lanes 6 and 7). This decrease was more apparent when the cells were treated with a combination of all three agents (fig 2, lane 8).

9-*cis*RA plus cig synergistically inhibit expression of COX-2 and c-Jun in Caco2 cells

COX-2 may thus play a critical role in the development of colorectal cancer.²¹ We therefore next examined whether the combined treatment with 9-*cis*RA plus cig inhibits the expression of COX-2 and c-Jun, a component of AP-1 which plays a role in the expression of COX-2 at the level of transcription,^{30, 31} in Caco2 colon cancer cells (fig 3). In the presence of PD98059, 9-*cis*RA or cig caused a small decrease in the levels of COX-2 (fig 3A, lanes 6 or 7, lower panel) and c-Jun (fig 3B, lanes 6 or 7, lower panel) proteins. The combination of these three agents further inhibited the expression of these proteins (fig 3A and B, lanes 8, lower panel). The decreases in COX-2 and c-Jun proteins were paralleled by similar decreases in their mRNAs (fig 3A and B, upper panels). Moreover, as shown in fig 3C and D, similar effects for the expression levels of both COX-2 and c-Jun proteins and mRNAs were also observed in mutant RAR α

T82A/S260A-transfected Caco2 cells which do not express the p-RXR α protein (data not shown). We previously reported that MAPKs are involved in RXR α phosphorylation in human HCC cells.^{11–15} Therefore, these findings suggest that the phosphorylation of the RXR α protein by MAPKs may thus interfere with the function of the ligands for RXR α and PPAR γ and that the induction of the unphosphorylated (ie, functional) form of RXR α intensifies the responses to ligands of RXR α and PPAR γ , thereby affecting the expression of target molecules, including COX-2 and c-Jun.

9-*cis*RA plus cig synergistically activate the PPRE promoter activity, inhibit the AP-1 promoter activity, inhibit cell growth and induce apoptosis in Caco2 cells

PPAR γ regulates gene expression by binding to the PPRE element in target genes as a heterodimer with RXRs and thus controlling growth of cells.⁸ We then examined the effects of the combination of 9-*cis*RA plus cig on the transcriptional activity of the PPRE and AP-1 promoters, using transient transfection luciferase reporter assays. As shown in fig 4A, left

panel, we found that in mutant RAR α T82A/S260A-transfected Caco2 cells, the treatment with this combination caused a synergistic increase in the transcriptional activity of the PPRE luciferase reporter activity. Treatment with either 9-*cis*RA alone or cig alone also caused significant inhibition of the AP-1 luciferase reporter activity, and this inhibition was thus enhanced when the cells were treated with a combination of these agents (fig 4A, right panel). Moreover, in the presence of PD98059, treatment with 9-*cis*RA or cig significantly inhibited the growth of Caco2 cells, and the combined treatment with all three agents caused even stronger growth inhibition (fig. 4B, lanes 6–8, left panel). Similar growth inhibitory effects by treatment with these ligands were also observed in mutant RAR α T82A/S260A-transfected Caco2 cells (fig 4B, lanes 6–8, right panel). In addition, as shown in fig 4C, the DNA fragmentation assays (left panel) and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) methods (right panel) indicated that, in the presence of PD98059, the combination of 9-*cis*RA plus cig caused a significant induction of apoptosis in Caco2 cells (lane 8 of left

Figure 3 Effects of the combination of 9-*cis*-retinoic acid (RA) plus ciglitazone (cig) on cellular levels of cyclo-oxygenase-2 (COX-2) mRNA and protein, and on c-Jun mRNA and protein in Caco2 cells. (A and B) The cells were treated with vehicle (lanes 1 and 5), 5 μ M 9-*cis*RA (lanes 2 and 6), 10 μ M cig (lanes 3 and 7) or the combination of 5 μ M 9-*cis*RA plus 10 μ M cig (lanes 4 and 8) for 24 h in the absence (lanes 1–4) or presence (lanes 5–8) of PD98059, and cell extracts were examined by a reverse transcription-PCR (RT-PCR) analysis using COX-2- (A, upper panel) or c-Jun- (B, upper panel) specific primers, or then were also examined by a western blot analysis using COX-2- (A, lower panel) or c-Jun- (B, lower panel) specific antibodies. (C and D) After Caco2 cells were transfected with wild-type (WT; lanes 1–4) or mutant RAR α (lanes 5–8), the cells were treated with vehicle (lanes 1 and 5), 5 μ M 9-*cis*RA (lanes 2 and 6), 10 μ M cig (lanes 3 and 7) and the combination of 5 μ M 9-*cis*RA plus 10 μ M cig (lanes 4 and 8) for 24 h. The cell extracts were then examined by a RT-PCR analysis using COX-2- (C, upper panel) or c-Jun- (D, upper panel) specific primers, or then were examined by a western blot analysis using COX-2- (C, lower panel) or c-Jun- (D, lower panel) specific antibodies. Amplified PCR products obtained with glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers served as internal controls (A–D, upper panels). Protein expression of GAPDH served as a loading control (A–D, lower panels). The intensities of the bands were quantitated by densitometry and the values are shown as a bar graph. Similar results were obtained in a repeat experiment. The asterisks represent a significant difference ($p < 0.01$) in comparison with columns 1 and 5. Bars show the SD.

panel and photograph 8 of right panel). These findings indicate that under conditions in which the phosphorylation of RXRα is reduced, the suppression of cell growth by combined treatment with 9-*cis*RA plus cig is associated with apoptosis.

The phosphorylated form of the RXRα protein is accumulated in human colorectal cancer tissues

We next examined whether the p-RXRα protein is present in human colorectal cancer tissues. Figure 5 shows the results of a western blot analysis of the nuclear extracts prepared from eight pairs of human colorectal cancer tissue and adjacent normal colon epithelial tissue. We thus found that the RXRα protein was highly phosphorylated in six of the eight samples of colorectal cancer tissues when compared with their corresponding normal colon epithelial tissue (fig 5), although there were no significant differences in the expression levels of the unphosphorylated form of RXRα proteins between cancer tissues and adjacent normal colon epithelial tissues (data not shown).

DISCUSSION

The present studies provide us with the first evidence that the RXRα protein is phosphorylated in both a series of human colon cancer cell lines and primary human colon cancer tissue samples, although the levels of expression of p-RXRα did not increase in FHC normal colonic epithelial cells (figs 1 and 5). These findings are consistent with our previous findings in human hepatoma cell lines and in hepatoma tissue samples,¹¹ and suggest that, as in hepatoma, a malfunction of RXRα due to phosphorylation might be associated with the development of colorectal cancer. In addition, we also found that the combination of 9-*cis*RA and cig caused a synergistic inhibition in the growth of human colon cancer Caco2 cells (fig 4B) and this was associated with the induction of apoptosis (fig 4C) and inhibition of the expression of both COX-2 and c-Jun proteins and mRNAs (fig 3). The combination of these agents had a synergistic effect in increasing the PPRE activity and decreasing the AP-1 activity (fig 4A). These preferable effects were observed when the phosphorylation of RXRα protein was either

Figure 4 Effects of the combination of 9-*cis*-retinoic acid (RA) plus ciglitazone (cig) on the transcriptional activity of the peroxisome proliferator-responsive element (PPRE) and AP-1 promoters (A), inhibition of cell growth (B) and induction of apoptosis (C) in Caco2 cells. (A) After Caco2 cells were transfected with wild-type (WT; lanes 1–4) or mutant retinoid X receptor α (RXRα) (lanes 5–8), transient transfection reporter assays were performed with the indicated luciferase reporter in the presence of vehicle (lanes 1 and 5), 5 μM 9-*cis*RA (lanes 2 and 6), 10 μM cig (lanes 3 and 7) or the combination of 5 μM 9-*cis*RA plus 10 μM cig (lanes 4 and 8). The relative luciferase activity was then determined after 24 h. (B) Left panel: Caco2 cells were treated with vehicle (lanes 1 and 5), 5 μM 9-*cis*RA (lanes 2 and 6), 10 μM cig (lanes 3 and 7) or the combination of 5 μM 9-*cis*RA plus 10 μM cig (lanes 4 and 8) for 24 h in the absence (lanes 1–4) or presence (lanes 5–8) of PD98059. (B) Right panel: after Caco2 cells were transfected with WT (lanes 1–4) or mutant forms of RXRα (lanes 5–8), the cells were treated with vehicle (lanes 1 and 5), 5 μM 9-*cis*RA (lanes 2 and 6), 10 μM cig (lanes 3 and 7) or the combination of 5 μM 9-*cis*RA plus 10 μM cig (lanes 4 and 8) for 24 h. The cell proliferation assay was then performed using the trypan blue dye exclusion method. Similar results were obtained in a repeat experiment. (C) Caco2 cells were treated with vehicle (lanes and photographs 1 and 5), 5 μM 9-*cis*RA (lanes and photographs 2 and 6), 10 μM cig (lanes and photographs 3 and 7) or the combination of 5 μM 9-*cis*RA plus 10 μM cig (lanes and photographs 4 and 8) for 48 h in the absence (lanes and photographs 1–4) or presence (lanes and photographs 5–8) of 50 μM PD98059, and the induction of apoptosis was determined by using the DNA fragmentation assays (left panel) and TUNEL assays (right panel). The asterisks represent a significant difference ($p < 0.01$) in comparison with columns 1 and 5. Bars show the SD of triplicate assays.

Figure 5 The expression levels of the phosphorylated form of the retinoid X receptor α (RXR α) protein in human colon cancer tissues. Eight pairs of human colon cancer (C) and its adjacent normal tissue (N) were obtained from surgical resections. Total protein was extracted and equivalent amounts of protein (15 μ g/lane) were examined by western blot analysis for the phosphorylated RXR α protein (p-RXR). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control.

inhibited by MEK inhibitor or transfected with point-mutated RXR α , which mimicked the unphosphorylated form. Therefore, the inhibition of the phosphorylation of this protein appears to play a critical role in inducing the growth inhibitory effect presented in this study.

A general question posed by the present study is the molecular mechanisms by which the combination of 9-*cis*RA and cig synergistically induce these diverse cellular and biochemical effects in colon cancer cells. A hypothetical scheme that addresses this question is shown in fig 6. In normal colonic epithelial cells, activated PPAR γ forms a heterodimer with

RXR α and binds to the PPRE in promoter regions of target genes.⁸ However, in colon cancer cells, the Ras/MAPK signalling pathway is highly activated and phosphorylates RXR α , thus impairing the functions of this receptor. The MEK inhibitor PD98059 or the point-mutated RXR α T82A/S260A prevent the phosphorylation of RXR α , thus causing an increase in the unphosphorylated form of RXR α protein and restoring the heterodimeric activity with PPAR γ in the presence of their ligands, 9-*cis*RA and cig. A synergistic effect on target gene expression was originally reported with the optimal interaction of RXR α -PPAR γ heterodimers.⁸ Tsao *et al*³² also reported that in HEK293 cells the transcriptional activity of PPRE was additively induced by treatment with a PPAR γ activator plus 9-*cis*RA, and RXR α accumulation through inhibiting its degradation by the proteasome system contributes to the enhancement of PPAR γ /RXR activation. It is of interest that the PPAR and RXR ligands have been shown to recruit subsets of transcriptional coactivators to the receptor complex differentially, thus leading to an enhanced transcriptional activation and cellular effects.³³ The findings of these reports support our finding that the accumulation of the unphosphorylated form (ie, functional form) of RXR α enhances the transcriptional activity of PPRE (fig 4A, left panel) and it may also explain the growth inhibitory effects of the combined treatment with RXR and PPAR ligands, which are observed in various types of cancer cells.¹⁷⁻²⁰

There is considerable evidence that COX-2 may play a critical role in the development of colorectal cancer and might, therefore, be an important molecular target for colorectal cancer prevention and treatment.^{21, 22} In this study, we found that the combined use of ligands for RXR α and PPAR γ inhibited COX-2 expression in Caco2 cells (fig 3A and C). This finding may be of significance because the inhibition of COX-2 expression is one of the mechanisms by which the PPAR γ pathway induces apoptosis.²⁰ Presumably, the inhibition of the transcriptional activity of the AP-1 promoter by the combined action of these agents (fig 4A, right panel) contributes to the inhibition of COX-2 expression because AP-1 plays an

Figure 6 A hypothetical schematic representation of the effects of the combination of 9-*cis*-retinoic acid (RA) plus ciglitazone (cig) on growth and gene expression in colon cancer cells. When peroxisome proliferator-activated receptor γ (PPAR γ) is activated by ligand binding, it is able to heterodimerise with retinoid X receptor (RXR) and activate target gene expression by binding to the peroxisome proliferator-responsive element (PPRE) in various genes in normal colonic epithelial cells (A). In colon cancer cells, the Ras/mitogen-activated protein kinase (MAPK) pathway phosphorylates RXR α , which impairs its function as a heterodimeric partner for PPAR γ (B). In the presence of MEK inhibitor PD98059, RXR α can escape phosphorylation by Erk (C). A point-mutated RXR α T82A/S260A, which mimics the unphosphorylated form of RXR α , can form a heterodimer with PPAR γ and thereby activate target gene expression by binding to the PPRE (D). Combined treatment with 9-*cis*RA plus cig can activate the transcriptional activity of PPRE and synergistically inhibit the expression of c-Jun, thus inhibiting the transcriptional activity of AP-1 and the expression of cyclo-oxygenase-2 (COX-2) in colon cancer cells (E). These combined effects may contribute to growth inhibition in colon cancer cells.

important role in activating the expression of this molecule.^{30, 31} However, it is likely that some other unknown mechanisms also play a role. For example, in the 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced rat colitis model, administration of a PPAR γ agonist reduced the expression of COX-2 and nuclear transcription factor NF- κ B p65 proteins.³⁴ Both NF- κ B and AP-1 play an important role in regulating COX-2 expression via the NF- κ B-binding site in the COX-2 promoter.³⁵ Moreover, TNBS-induced colitis was significantly reduced by the administration of both PPAR γ and RXR agonists, and this beneficial effect was also reflected by the reduction in the NF- κ B DNA binding activity in the colon.³⁶ PPAR γ agonist has been shown to inhibit chemically induced colitis and an early phase of colitis-related colon carcinogenesis in the rodent model.³⁷ It also seems to be of interest that the inhibition of the β -catenin pathway, which promotes the development of colon cancer, by non-steroidal anti-inflammatory drugs, requires a high level of expression of RXR α and PPAR γ .³⁸ As a result, the activation of the RXR-PPAR γ heterodimer by the co-administration of their ligands is thus considered to protect against colonic inflammation and tumorigenesis. In conclusion, these previous reports, together with the results of our in vitro mechanistic studies on colon cancer cells as described herein, suggest that combination therapy with both RXR and PPAR γ ligands might therefore be clinically useful in the prevention and/or treatment of both colon cancer and colonic inflammation, due to their synergistic effects.³⁹

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