

# Down-regulation of X-linked inhibitor of apoptosis synergistically enhanced peroxisome proliferator-activated receptor $\gamma$ ligand-induced growth inhibition in colon cancer

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## Abstract

We found previously that X-linked inhibitor of apoptosis protein (XIAP), a potent endogenous inhibitor of apoptosis, is overexpressed in colon cancer. Ligand-induced activation of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) has been shown to exert proapoptotic and anti-proliferative effects in many cancer cell types. However, neither XIAP down-regulation alone nor monotherapy using PPAR $\gamma$  ligands is potent enough to control colon cancer. We explored whether XIAP inhibition and PPAR $\gamma$  activation offer a synergistic anticancer effect in colon cancer. HCT116-XIAP<sup>+/+</sup> and HCT116-XIAP<sup>-/-</sup> cells were treated with troglitazone or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15-PGJ<sub>2</sub>). Cell growth and apoptosis were measured. Nude mice were s.c. inoculated with HCT116 cells with or without oral troglitazone. Tumor growth, angiogenesis, and apoptosis were measured. Troglitazone- and 15-PGJ<sub>2</sub>-induced growth inhibition and apoptosis were more prominent in HCT116-XIAP<sup>-/-</sup> cells. Troglitazone- and 15-PGJ<sub>2</sub>-induced apoptosis correlated with enhanced cleavage of caspases and poly(ADP-ribose) polymerase, which were more profound in HCT116-XIAP<sup>-/-</sup> cells. Pretreatment of cells with XIAP inhibitor 1396-12 also sensitized HCT116-XIAP<sup>+/+</sup> cells to PPAR $\gamma$  ligand-induced apoptosis. Troglitazone significantly retarded the growth of xenograft tumors, more significantly so in HCT116-XIAP<sup>-/-</sup> cell-derived tumors. Reduction of tumor size was associated with reduced expression of Ki-67,

vascular endothelial growth factor, and CD31 as well as increased apoptosis. Loss of XIAP significantly sensitized colorectal cancer cells to PPAR $\gamma$  ligand-induced apoptosis and inhibition of cell proliferation. Thus, simultaneous inhibition of XIAP and activation of PPAR $\gamma$  may have a synergistic antitumor effect against colon cancer. [Mol Cancer Ther 2008;7(7):2203–11]

## Introduction

X-linked inhibitor of apoptosis protein (XIAP) is the most potent endogenous inhibitor of apoptosis. XIAP exerts its antiapoptotic effect through inactivating key caspases such as caspase-3, caspase-7, and caspase-9. Overexpression of XIAP was associated with treatment resistance (1, 2), whereas down-regulation of XIAP makes cancer cells more vulnerable to various apoptosis-inducing agents and conditions such as TRAIL and hypoxia (3, 4). Inhibition of XIAP also reduces angiogenesis and suppresses tumorigenicity (5, 6). Thus, XIAP has a great potential in cancer gene therapy.

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a transcription factor richly expressed in many tissues including normal colonic mucosa and colon cancers. It can be activated by many endogenous or natural ligands [such as prostanoids, prostaglandin D<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), and certain polyunsaturated fatty acids] and exogenous or synthetic ligands (such as troglitazone, rosiglitazone, ciglitazone, and pioglitazone; ref. 7). Binding of these ligands to PPAR $\gamma$  receptor results in heterodimerization of PPAR $\gamma$  with the retinoid X receptor, which then binds to PPAR $\gamma$  peroxisome response elements, leading to transcription of downstream target genes (7, 8).

Many *in vitro* studies have indicated that ligand-induced activation of PPAR $\gamma$  induces cell differentiation and apoptosis in several cancer cell types (9–13). PPAR $\gamma$  activation was also found to inhibit angiogenesis in many human cancers (14). Many *in vivo* studies have revealed that ligand-induced activation of PPAR $\gamma$  not only reduces the precursor lesions of colorectal cancer (15) but also suppresses colorectal cancer growth in mice (9, 10, 12, 16). Mice with heterozygous loss of PPAR $\gamma$  are more susceptible to carcinogen-induced and spontaneous carcinogenesis of the colon and mammary gland (17–19). Despite these reports, the therapeutic efficacy of PPAR $\gamma$  ligands for cancers is very limited. Some phase II clinical trials using troglitazone in patients with metastatic colorectal cancer did not show any impressive therapeutic efficacy (20, 21). Furthermore, activation of PPAR $\gamma$  has

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produced controversial results in colon cancer (19–22). For example, activation of PPAR $\gamma$  by its ligands promoted rather than suppressed colorectal cancer growth (19–25). Thus, the role of PPAR $\gamma$  in the pathogenesis of colorectal cancer has not been clearly defined (26), and the use of PPAR $\gamma$  ligands as a potential chemopreventive and a therapeutic measure for colorectal cancer still requires intensive research.

Based on the fact that the antiapoptotic protein XIAP is generally overexpressed in many solid tumors including colon cancer, and PPAR $\gamma$  is critically involved in the regulation of colonic epithelial growth and apoptosis, we hypothesized that simultaneous down-regulation of XIAP and activation of PPAR $\gamma$  may offer a synergistic therapeutic effect against colorectal cancer. Thus far, there have been very few studies addressing the synergistic role of XIAP and PPAR $\gamma$  in colorectal cancer. In this study, we conducted a series of *in vitro* and *in vivo* experiments using colon cancer cell line HCT116 cells as a model and two most commonly used ligands troglitazone and 15d-PGJ<sub>2</sub> as potent activators for PPAR $\gamma$  to test our hypothesis.

## Materials and Methods

### Chemicals, Reagents, and Cell Lines

15-PGJ<sub>2</sub> and troglitazone were purchased from Cayman Chemicals. XIAP-specific inhibitor 1396-12 was a gift from

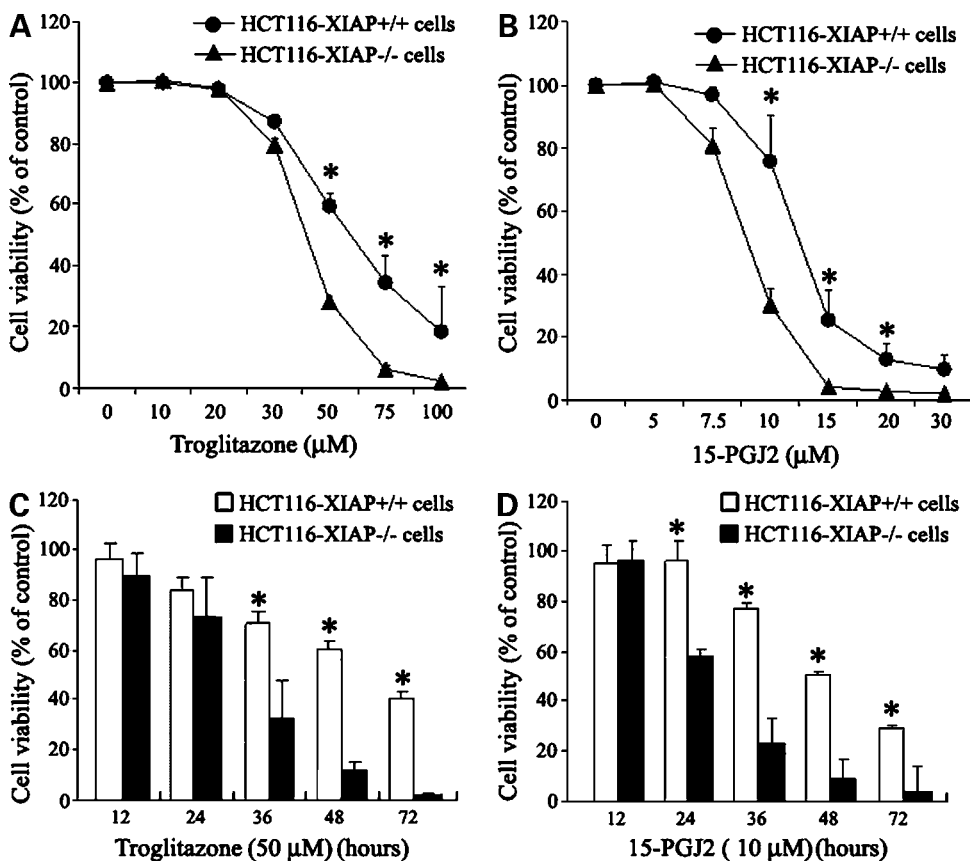
Dr. Clemencia Pinilla (Torrey Pines Institute for Molecular Studies; ref. 27). Human colon cancer cell lines HCT116-XIAP<sup>-/-</sup>, HCT116-XIAP<sup>+/+</sup>, DLD1-XIAP<sup>-/-</sup>, and DLD1-XIAP<sup>+/+</sup> were gifts from Prof. Bert Vogelstein (Howard Hughes Medical Institute; ref. 28). Other colon cancer cell lines were obtained from the American Type Culture Collection. The proteasome inhibitors MG132 and lactacystin were obtained from Calbiochem. Trizol, PCR-related reagents, and all cell culture-related materials were purchased from Invitrogen. WST-1 reagent, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit, and Cell Death Detection ELISA<sup>PLUS</sup> Assay kit were purchased from Roche Diagnostics. Anti-XIAP was purchased from MBL International (Woburn). Anti-glyceraldehyde-3-phosphate dehydrogenase and anti-Ki-67 were purchased from Abcam. All other primary and secondary antibodies were purchased from Santa Cruz Biotechnology.

### Cell Culture and Viability Assay

HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and maintained in a humidified incubator at 37°C in a 5% CO<sub>2</sub> atmosphere. Cell viability was determined by WST-1 assay as reported (29).

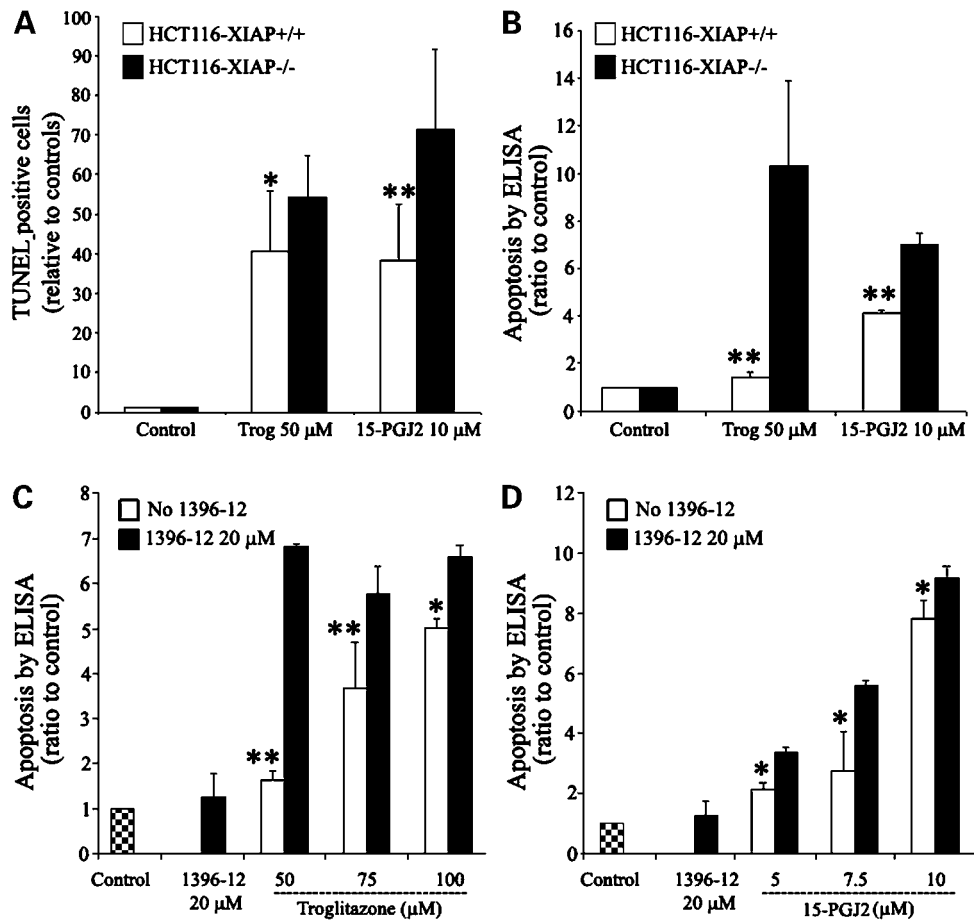
### Reverse Transcription-PCR

Total RNA was extracted using Trizol reagent. Total RNA (1  $\mu$ g) was reverse transcribed into cDNA and reverse



**Figure 1.** Effects of troglitazone and 15-PGJ<sub>2</sub> on cell viability. HCT116-XIAP<sup>+/+</sup> and HCT116-XIAP<sup>-/-</sup> cells were treated for 48 h with various concentrations of troglitazone (A) and 15-PGJ<sub>2</sub> (B) or with 50  $\mu$ mol/L troglitazone (C) or 10  $\mu$ mol/L 15-PGJ<sub>2</sub> (D) for various durations. Mean  $\pm$  SD of three separate experiments each with three replicates. \*,  $P < 0.001$ , compared with HCT116-XIAP<sup>+/+</sup> cells.

**Figure 2.** Effect of loss of XIAP on PPAR $\gamma$  ligand-induced apoptosis. HCT116-XIAP $^{+/+}$  and HCT116-XIAP $^{-/-}$  cells were exposed to 50  $\mu$ mol/L troglitazone or 10  $\mu$ mol/L 15-PGJ $_2$  for 48 h. Apoptosis was determined by TUNEL (A) and cell death ELISA assay (B). In separate studies, HCT116-XIAP $^{+/+}$  cells were treated with various concentrations of troglitazone (C) and 15-PGJ $_2$  (D) in the presence or absence of 20  $\mu$ mol/L 1396-12 for 48 h and apoptosis was determined by ELISA assay. Mean  $\pm$  SD of three replicates. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ , compared with HCT116-XIAP $^{-/-}$  cells.



transcription-PCR was done using the following primers: human glyceraldehyde-3-phosphate dehydrogenase sense 5'-TGCCTCCTGCACCACCAACT-3' and antisense 5'-CCCCTCAGCTCAGGGATGA-3', human XIAP sense 5'-AGGAACCCTGCCATGTATTG-3' and antisense 5'-CACT TCTGGGAACCCTTGTT-3', and human PPAR $\gamma$  sense 5'-TCTCTCCGTAATGGAAGACC-3' and antisense 5'-GCATTATGAGACATCCCCAC-3 as we have described previously (30).

#### Western Blotting

Total protein was extracted in radioimmunoprecipitation assay lysis buffer supplemented with 1% of protease inhibitor cocktail. Protein (25  $\mu$ g) was separated via 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). Western blot was done as we have reported (29–31).

#### Detection of Apoptosis in Cultured Cells

Apoptosis was analyzed by TUNEL assay and Cell Death Detection ELISA<sup>PLUS</sup> Assay. TUNEL was done as reported previously (29, 31). For Cell Death Detection ELISA<sup>PLUS</sup> Assay,  $1 \times 10^4$  cells per well were cultured, treated in a 96-well plate, and disrupted in lysis buffer, and the microplate was centrifuged at  $200 \times g$ . The supernatant (20  $\mu$ L) was incubated with 80  $\mu$ L immunoreagent containing anti-histone-biotin and anti-DNA-peroxidase in a streptavidin-

coated microplate for 2 h at room temperature. Color was developed with substrate solution for 15 min and the absorbance at 405 nm was taken. DNA fragmentation was expressed as the ratio of absorbance of treated to control cells.

#### Coimmunoprecipitation

Cells were disrupted in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100] containing 1% protease inhibitor cocktail. Supernatant was collected after centrifugation at  $10,000 \times g$  for 20 min at 4°C. Total protein (500  $\mu$ g) was incubated with 30  $\mu$ L protein G-agarose beads for 1 h at 4°C followed by incubation with 1  $\mu$ g anti-XIAP antibody at 4°C overnight. After washing, the beads were resuspended in Laemmli buffer, boiled, and eluted. The eluted protein was subjected to SDS-PAGE and probed with anti-ubiquitin antibody.

#### Effect of Troglitazone on Xenograft Colon Cancers in Nude Mice

Use of animals was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

To generate xenograft colon cancers,  $1 \times 10^7$  HCT116-XIAP $^{+/+}$  or HCT116-XIAP $^{-/-}$  cells in 100  $\mu$ L sterile PBS were s.c. injected into the right dorsal flanks of nude mice.

For prevention study, animals received daily troglitazone in diet (200 ppm;  $n = 6$ ) or vehicle ( $n = 6$ ) from the day of tumor cell injection, and experiments continued for 33 days. For regression study, mice were injected with tumor cells as stated above. When the tumors reached  $\sim 5$  mm in diameter, animals were given daily troglitazone or vehicle, and experiments continued for 24 days. In all groups, tumor volumes were measured every other day as we have reported (31). At harvest, tumors were removed, size was measured, and weight was determined.

#### Expression of Ki-67 and Vascular Endothelial Growth Factor in Tumors

Harvested tumor tissues were homogenized in lysis buffer and Western blot was done to detect the expressions of Ki-67 and vascular endothelial growth factor. Expression of CD31 was detected by standard immunohistochemistry in the tumor tissues. Mitotic figures were counted in H&E slides.

#### Detection of Apoptosis in Tumors by TUNEL

Tumor tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4  $\mu$ m sections. TUNEL assay was done in tissue slides as described (31), except

that TUNEL-positive cells were analyzed with a fluorescence microscope.

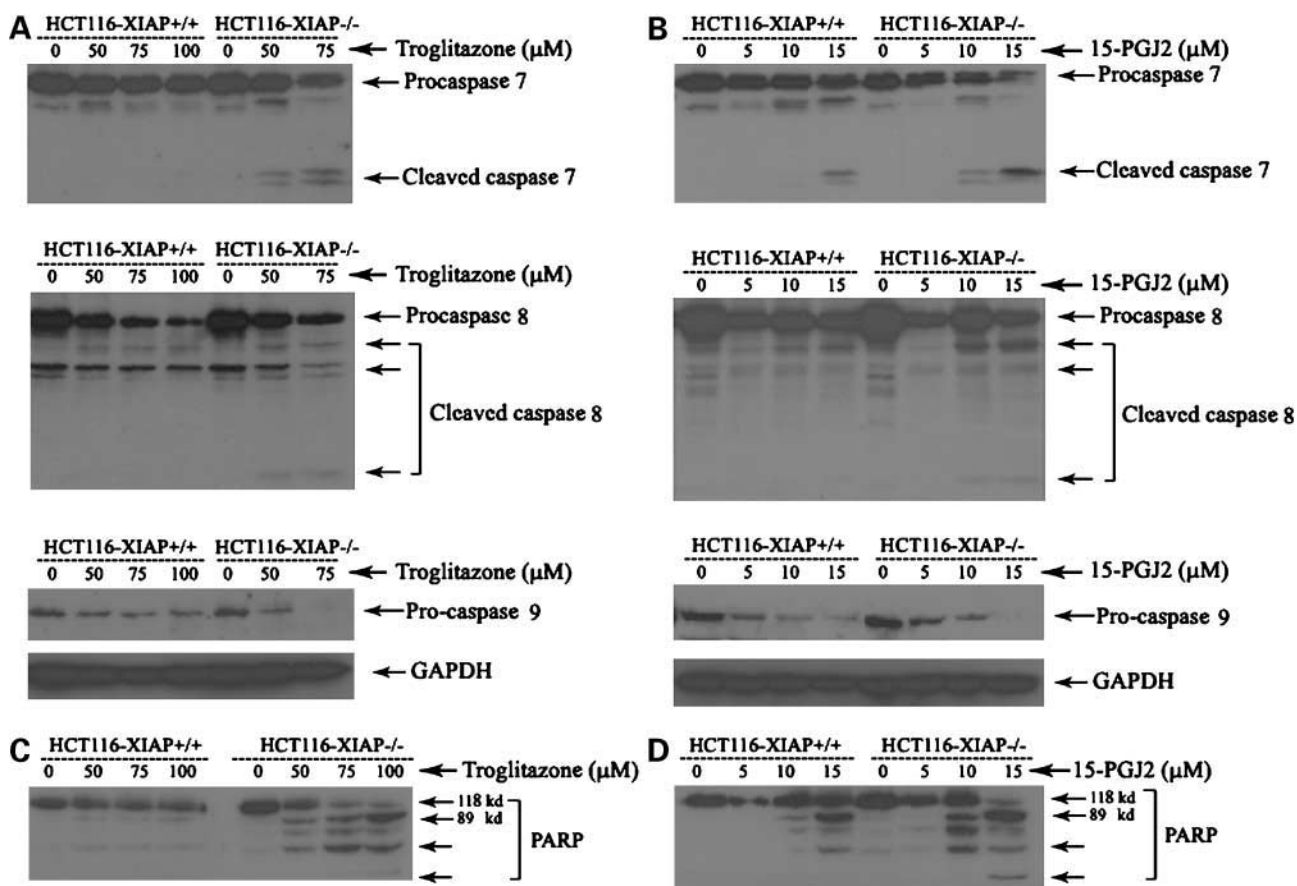
#### Statistical Analysis

SPSS (version 13.0) was used to conduct the statistical analysis. One-way ANOVA was used to examine the differences between each treatment groups.  $P < 0.05$  was considered statistically significant.

## Results

#### Expression of XIAP and PPAR $\gamma$ in Colon Cancer Cell Lines

We first detected the expression levels of XIAP and PPAR $\gamma$  in seven colon cancer cells: SW480, SW1116, LOVO, HT29, HCT15, HCT116, and DLD1. All these cell lines expressed ample amount of XIAP and PPAR $\gamma$  (data not shown). In HCT116 and DLD1 cells, we detected the expression of these two genes in cells with two different XIAP genotypes. XIAP and PPAR $\gamma$  were readily detectable in HCT116-XIAP $^{+/+}$  and DLD1-XIAP $^{+/+}$  cells. In contrast, XIAP was not detectable and PPAR $\gamma$  was down-regulated in HCT116-XIAP $^{-/-}$  and DLD1-XIAP $^{-/-}$  cells (data not shown).



**Figure 3.** Effects of troglitazone and 15-PGJ $_2$  on apoptosis-related proteins. HCT116-XIAP $^{+/+}$  and HCT116-XIAP $^{-/-}$  cells were treated with various concentrations of troglitazone (A and C) and 15-PGJ $_2$  (B and D) for 48 h. Expressions of caspase-7, caspase-8, and caspase-9 (A and B) and poly(ADP-ribose) polymerase (C and D) were detected by Western blot.

### Loss of XIAP Rendered HCT116 Cells More Susceptible to PPAR $\gamma$ Ligand-Induced Growth Inhibition and Apoptosis

We treated the HCT116-XIAP<sup>+/+</sup> and HCT116-XIAP<sup>-/-</sup> cells with various concentrations of two PPAR $\gamma$ -specific ligands troglitazone and 15-PGJ<sub>2</sub>. As shown in Fig. 1, troglitazone and 15-PGJ<sub>2</sub> caused a significant loss of cell viability in a dose-dependent (Fig. 1A and B) and time-dependent (Fig. 1C and D) manner. Such a growth inhibition was more profound in HCT116-XIAP<sup>-/-</sup> cells.

Inhibition of cell proliferation was associated with induction of apoptosis. As revealed by TUNEL assay shown in Fig. 2A, troglitazone and 15-PGJ<sub>2</sub> induced apoptosis in both cell types. Consistently, there was also a marked increase in DNA fragmentation (Fig. 2B), which also indicates that HCT116-XIAP<sup>-/-</sup> cells are more susceptible than HCT116-XIAP<sup>+/+</sup> cells to PPAR $\gamma$  agonist-induced apoptosis.

Similar results were found in DLD1-XIAP<sup>+/+</sup> and DLD1-XIAP<sup>-/-</sup> cells (data not shown).

### Effect of XIAP Inhibitor on Troglitazone- and 15-PGJ<sub>2</sub>-Induced Cell Killing

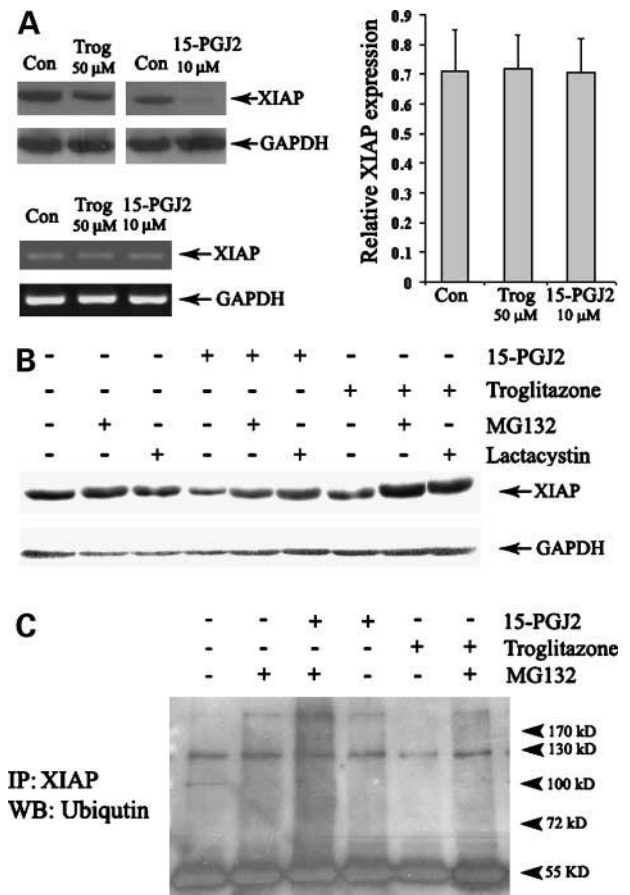
To further confirm whether loss of XIAP function could sensitize HCT116 cells to PPAR $\gamma$  ligand-induced apoptosis, HCT116-XIAP<sup>+/+</sup> cells were treated with various concentration of troglitazone and 15-PGJ<sub>2</sub> for 48 h in the presence or absence of 20  $\mu$ mol/L 1396-12, a specific XIAP inhibitor. As shown in Fig. 2C and D, 1396-12 markedly sensitized cells to troglitazone and 15-PGJ<sub>2</sub>-induced apoptosis.

### Loss of XIAP Led to Enhanced Cleavage of Caspases and Poly(ADP-Ribose) Polymerase in HCT116 Cells

We then studied the effect of loss of XIAP on PPAR $\gamma$  ligand-induced caspase cleavage. As shown in Fig. 3, troglitazone (Fig. 3A) and 15-PGJ<sub>2</sub> (Fig. 3B) dose-dependently induced a cleavage of caspase-7, caspase-8, and caspase-9. The ligand-induced caspase cleavage was associated with increased poly(ADP-ribose) polymerase cleavage (Fig. 3C and D). Cleavage of caspases and poly(ADP-ribose) polymerase are more prominent in HCT116-XIAP<sup>-/-</sup> cells.

### Troglitazone and 15-PGJ<sub>2</sub> Induced Proteasomal Degradation of XIAP

Ubiquitination and proteasomal-mediated degradation of antiapoptotic proteins is one of the mechanisms of certain anticancer drugs. We noticed a marked reduction of XIAP protein expression following PPAR $\gamma$  ligand treatment (Fig. 4A). Both troglitazone- and 15-PGJ<sub>2</sub>-induced reduction of XIAP occurred at the translational level (Fig. 4A, top left) but not transcriptional level (Fig. 4A, bottom left and right). The ligand-induced down-regulation of XIAP was consistently associated with increased apoptosis (data not shown). We speculated that reduced XIAP expression in cells exposed to troglitazone and 15-PGJ<sub>2</sub> may have resulted from the ligand-induced activation of proteasomal degradation. We therefore used two commonly used proteasome inhibitors MG132 and lactacystin to test this speculation. Troglitazone- and 15-PGJ<sub>2</sub>-induced XIAP down-regulation was reversed by MG132 and lactacystin



**Figure 4.** Down-regulation of XIAP by PPAR $\gamma$  ligands via proteasomal degradation. HCT116-XIAP<sup>+/+</sup> cells were treated with troglitazone (50  $\mu$ mol/L) and 15-PGJ<sub>2</sub> (10  $\mu$ mol/L) for 48 h, and the expression of XIAP was detected by Western blot (A, top left) and reverse transcription-PCR (A, bottom left and right). In separate studies, HCT116-XIAP<sup>+/+</sup> cells were pretreated with MG132 (25  $\mu$ mol/L) and lactacystin (20  $\mu$ mol/L) for 2 h followed by 50  $\mu$ mol/L troglitazone or 10  $\mu$ mol/L 15-PGJ<sub>2</sub> for 48 h (B). In a coimmunoprecipitation assay, HCT116-XIAP<sup>+/+</sup> cells were pretreated with or without MG132 (25  $\mu$ mol/L) and troglitazone (50  $\mu$ mol/L) or 15-PGJ<sub>2</sub> (10  $\mu$ mol/L) for 48 h. Whole-cell lysate was immunoprecipitated using anti-XIAP and then probed with anti-ubiquitin (C).

(Fig. 4B). Furthermore, a coimmunoprecipitation assay showed that troglitazone and 15-PGJ<sub>2</sub> enhanced the expression of ubiquitylated XIAP in the presence of MG132 (Fig. 4C). These results indicate that troglitazone and 15-PGJ<sub>2</sub> induced XIAP degradation via promoting its ubiquitination.

### Loss of XIAP Enhanced the Inhibitory Effect of Troglitazone on Xenograft Colon Cancers

The above studies indicated that PPAR $\gamma$  ligands exert antiproliferation and proapoptotic effects in colon cancer cells, and loss of XIAP could greatly enhance these effects. We then tested these observations *in vivo* using troglitazone.

All animals injected with HCT116-XIAP<sup>+/+</sup> and HCT116-XIAP<sup>-/-</sup> cells developed visible tumors 3 days

after inoculation, and the overall growth speed did not differ between these two groups of tumors. However, tumors in animals receiving troglitazone grew much slower than those mice without troglitazone (Fig. 5). In prevention study, HCT116-XIAP<sup>+/+</sup> cell-derived tumors borne by mice receiving troglitazone grew much slower than tumors borne by mice receiving control diet (no-troglitazone; Fig. 5A). At harvest, the average tumor weight in troglitazone group was 54% lighter than that of the no-troglitazone group (Fig. 5B; 0.15 versus 0.33 g;  $P < 0.01$ ).

In contrast, loss of XIAP significantly enhanced troglitazone-induced tumor suppression. HCT116-XIAP<sup>-/-</sup> cell-derived tumors in mice receiving troglitazone grew significantly slower than tumors in mice without troglitazone (no-troglitazone; Fig. 5C). At harvest, the average tumor weight of the troglitazone group was 79% lighter than that of the no-troglitazone group (Fig. 5D; 0.07 versus 0.33 g, respectively;  $P < 0.001$ ).

Similarly in regression study, tumor growth in the troglitazone-treated mice was much slower than that in mice without troglitazone (no-troglitazone). At harvest, the average tumor weight of the troglitazone group was only 53% of that of the no-troglitazone group (0.46 versus 0.87 g;  $P < 0.01$ ).

#### Expression of Vascular Endothelial Growth Factor and Ki-67 and Apoptosis in Xenograft Tumors

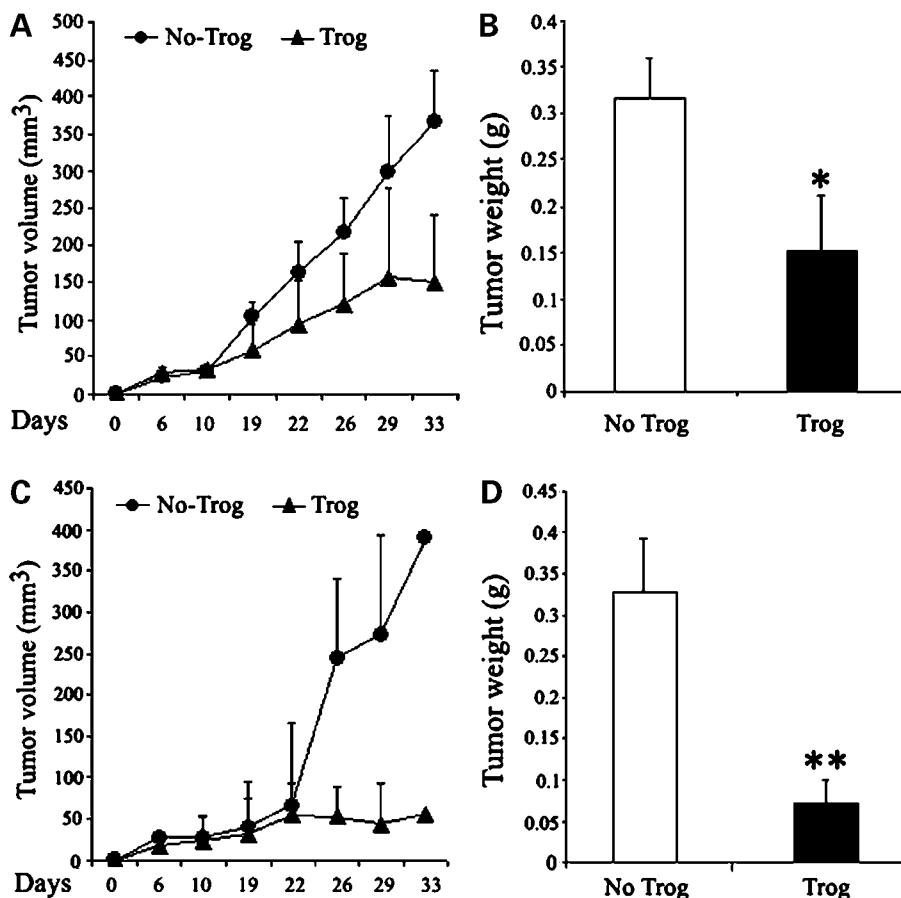
In HCT116-XIAP<sup>+/+</sup> cell-derived tumors, administration of troglitazone resulted in a decreased expression of Ki-67 (Fig. 6A), although the changes did not reach statistical significance. In contrast, administration of troglitazone led to a significantly decreased expression of Ki-67 (Fig. 6B) in HCT116-XIAP<sup>-/-</sup> cell-derived tumors. Troglitazone also caused a reduction of CD31, vascular endothelial growth factor, and the number of mitotic figures, especially in HCT116-XIAP<sup>-/-</sup> cell-derived tumors (data not shown).

No difference was observed in the basal expressions of Ki-67 and vascular endothelial growth factor between HCT116-XIAP<sup>-/-</sup> and HCT116-XIAP<sup>+/+</sup> cell-derived tumors and between HCT116-XIAP<sup>+/+</sup> cells derived tumors treated with or without troglitazone.

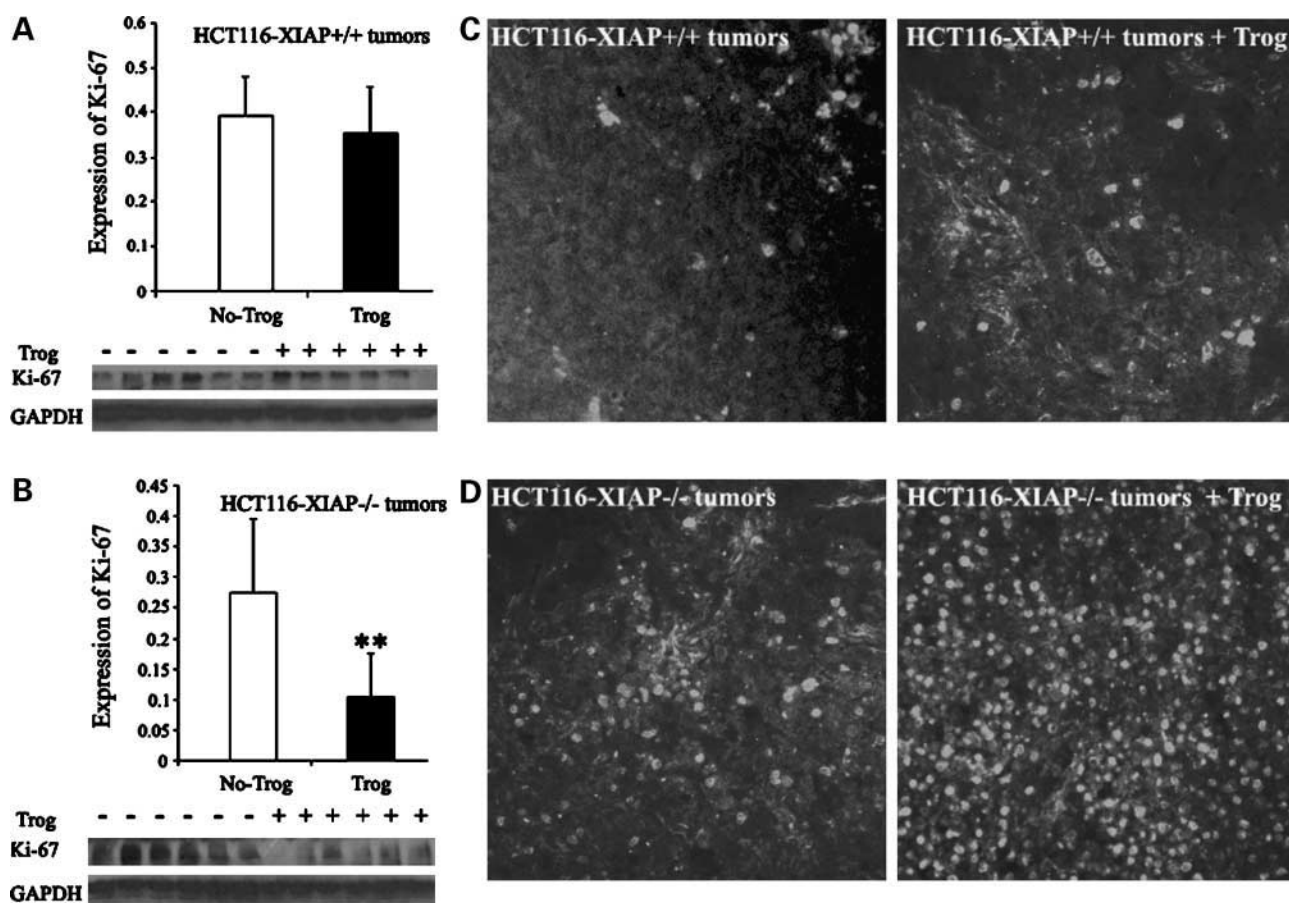
In addition, troglitazone led to increased apoptosis in xenograft tumors (Fig. 6C and D). In particular, a large number of TUNEL-positive cells (>30%) were observed in HCT116-XIAP<sup>-/-</sup> cell-derived tumors (Fig. 6D).

#### Discussion

In this study, we showed that XIAP and PPAR $\gamma$  were abundantly expressed in colon cancer cells and loss of



**Figure 5.** Effects of troglitazone on the growth of xenograft colon cancers. In prevention study (A-D), 12 nude mice were s.c. inoculated with HCT116-XIAP<sup>+/+</sup> cells (A and B) or HCT116-XIAP<sup>-/-</sup> cells (C and D). Mice were then given oral troglitazone ( $n = 6$ ) or vehicle ( $n = 6$ ). A and B, tumor growth velocity over a period of 33 days. C and D, average tumor weight at harvest. Mean  $\pm$  SD. \*,  $P < 0.01$ , troglitazone versus no-troglitazone; \*\*,  $P < 0.001$ , troglitazone versus no-troglitazone.



**Figure 6.** Effects of troglitazone on expression of Ki-67 (**A** and **B**) and apoptosis (**C** and **D**). Expression of Ki-67 was detected by Western blot in tissue lysates from HCT116-XIAP<sup>+/+</sup> (**A**) and HCT116-XIAP<sup>-/-</sup> (**B**) cell-derived tumors treated with (troglitazone) or without troglitazone (no-troglitazone). Mean  $\pm$  SD ( $n = 6$ ). \*\*,  $P < 0.01$ , between troglitazone and no-troglitazone groups. Effect of troglitazone on apoptosis was detected by TUNEL assay in sections of xenograft tumors derived from HCT116-XIAP<sup>+/+</sup> cells (**C**) and HCT116-XIAP<sup>-/-</sup> cells (**D**) in mice with or without troglitazone.

XIAP expression was associated with reduced PPAR $\gamma$  expression. We also observed that the transcription activity of PPAR $\gamma$  is reduced in colon cancer.<sup>4</sup> Previous studies by others suggested that down-regulation of XIAP possessed anticancer effects (5, 6). Similarly, PPAR $\gamma$  ligands and ligand-induced activation of PPAR $\gamma$  also exhibited antitumor effects (15, 16). However, neither of these approaches alone was potent enough for cancer therapy. Thus, we reasoned that simultaneous down-regulating XIAP and activating PPAR $\gamma$  might hold a potential for colon cancer therapy.

We found that treatment of HCT116-XIAP<sup>+/+</sup> and HCT116-XIAP<sup>-/-</sup> cells with two PPAR $\gamma$  ligands led to a suppression of cell proliferation, more dramatically so in HCT116-XIAP<sup>-/-</sup> cells. This finding was consistent with previous studies that down-regulation of XIAP enhanced TRAIL-induced and other drug-induced cell killing in several cancer cell lines (3, 4, 27, 32, 33).

It was reported recently that XIAP knocking down per se potentially induces apoptosis in glioma cells and estrogen-dependent breast cancer cells (34, 35). Our study showed that, in HCT116 cells, knocking down of XIAP by itself is not potent enough to enhance spontaneous apoptosis. Knocking down of XIAP, however, can dramatically sensitize HCT116 cells to apoptosis in response to PPAR $\gamma$  ligands, further confirming that PPAR $\gamma$  ligand-induced killing of HCT116 cells was mainly through apoptosis, and XIAP is a critical determinant for the sensitivity of this cell type to undergo apoptosis.

Caspases are key players in apoptosis. We found that troglitazone and 15-PGJ<sub>2</sub> induced marked cleavage of caspase-7, caspase-8, and caspase-9 in HCT116 cells. Cleavage of caspases was much more dramatic in HCT116-XIAP<sup>-/-</sup> cells than in HCT116-XIAP<sup>+/+</sup> cells. Furthermore, poly(ADP-ribose) polymerase, a well-known substrate for caspase-3, was also markedly cleaved, more so in HCT116-XIAP<sup>-/-</sup> cells.

Previous studies showed that cells expressing higher level of PPAR $\gamma$  were more sensitive to PPAR $\gamma$  ligands

<sup>4</sup> Our unpublished data.

(32, 36). We noted that PPAR $\gamma$  is expressed at relatively lower-level HCT116-XIAP $^{-/-}$  cells; yet, they are more sensitive to troglitazone and 15-PGJ $_2$  compared with HCT116-XIAP $^{+/+}$  cells. Thus, XIAP plays a major role in mediating cellular sensitivity to PPAR $\gamma$  ligands.

Certain anticancer drugs exert their effect through induction of proteasomal-mediated degradation of anti-apoptotic proteins (37). XIAP has ubiquitin protease ligase activity and its autoubiquitination and degradation is an important mechanism for regulating its function in apoptosis (38, 39). We found that troglitazone- and 15-PGJ $_2$ -induced reduction of XIAP was reversed by MG132 and lactacystin, whereas treatment of XIAP $^{+/+}$  cells with troglitazone or 15-PGJ $_2$  in the presence of MG132 increased the expression level of ubiquitylated XIAP. These results show that activation of the proteasome pathway and ubiquitin-mediated degradation are mechanistically responsible for XIAP reduction in troglitazone- and 15-PGJ $_2$ -treated cells. However, as the proteasomal inhibition may cause other nonspecific intracellular changes, the mechanisms involving PPAR $\gamma$  ligand-induced proteasomal degradation of XIAP requires further investigation. We have preliminary data suggesting the PPAR $\gamma$  ligand-induced nuclear factor- $\kappa$ B activation may have played a role in this process. Further studies are needed to better clarify this aspect.

Studies in nude mice showed that loss of XIAP did not affect the carcinogenicity of HCT116 cells, and tumors derived from HCT116-XIAP $^{+/+}$  and HCT116-XIAP $^{-/-}$  cells grew at a similar speed. These results are similar to the previous study in estrogen-independent breast cancer cells (33) but are different from a recent study in an estrogen-dependent breast cancer cell line (34), which showed that MCF7 cells with RNA interference-induced knocking down of XIAP nearly lost their tumorigenicity in nude mice. This difference may be due to several reasons: cell type specificity, number of tumor cells inoculated into mice, and "off-target" effect of gene knockdown.

In mice receiving troglitazone for 33 days, tumor growth was markedly suppressed. Such a suppression was strikingly more significant in tumors derived from HCT116-XIAP $^{-/-}$  cells, suggesting that loss of XIAP sensitizes these tumors to troglitazone-induced tumor suppression. Furthermore, troglitazone not only suppresses the development of xenograft tumors but also greatly slows down the progression of established tumors. These findings are consistent with our *in vitro* results that loss of XIAP significantly sensitizes HCT116 cells to troglitazone-induced growth suppression.

As orthotopic models of colon cancer more closely mimic the *in vivo* feature of colon cancer than the s.c. xenograft model (40), further studies using orthotopic colon cancer model would confirm our current results with more convincing data.

As XIAP is related to tumor angiogenesis (41) and PPAR $\gamma$  ligands could inhibit tumor growth via inhibition of angiogenesis (14), combination of loss of XIAP function and PPAR $\gamma$  ligands could achieve a synergistic anticancer effect by promoting apoptosis and inhibition of angiogen-

esis. Our finding that inhibition of vascular endothelial growth factor and CD31 was more prominent in HCT116-XIAP $^{-/-}$  cell-derived tumors treated with troglitazone appears to support this notion.

In conclusion, simultaneous down-regulation of XIAP and ligand-induced activation of PPAR $\gamma$  render colon cancer cells more susceptible to undergo apoptosis and growth inhibition. Therefore, such a combination may have a great potential in colon cancer therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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