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The 5-aminosalicylic acid antineoplastic effect in the intestine is mediated by PPARγ

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Epidemiological evidences suggested that 5-aminosalicylic acid (5-ASA) therapy may prevent the development of colorectal cancer in inflammatory bowel disease patients. Our aim is to investigate whether peroxisome proliferator-activated receptor-γ (PPARγ) mediates the antineoplastic effects of 5-ASA. HT-29 and Caco-2 cells were treated by 5-ASA, rosiglitazone (PPARγ ligand) or etoposide (anticarcinogenic drug). Epithelial cell growth, proliferation and apoptosis were assessed by cell count, Ki-67 staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, respectively. The antineoplastic effect of 5-ASA was evaluated in a xenograft tumor model in severe combined immunodeficiency (SCID) mice and in azoxymethane (AOM)-induced colon carcinogenesis in A/JOlaHsd mice. The role of PPARγ was examined by administration of PPARγ antagonist, GW9662 and in PPAR knockdown cells. Compared with untreated cells, treatment of HT-29 cells by 5-ASA inhibited significantly cell growth and cell proliferation (respectively, 60% and 63%) and induced apoptosis in 75% of cells. These effects were abolished by co-treatment with GW9662 and blunted in PPAR knockdown cells. Contrarily to etoposide, similar inhibitory effects of GW9662 were obtained in HT-29 cells treated with rosiglitazone. In the xenograft model, GW9662 abolished the therapeutic effect of 5-ASA, which decreased tumor weight and volume by 80% in SCID mice compared with untreated mice. In A/JOlaHsd mice, 5-ASA suppressed colon carcinogenesis by decreasing the number of aberrant crypt foci (75%) and aberrant crypts (22%) induced by AOM treatment with an absence of 5-ASA response after GW9662 administration. In conclusion, 5-ASA exerts potent antineoplastic effects that are mediated through PPARγ. These data provide new rational for designing more effective and safe antineoplastic PPARγ ligands with topical effects.

Introduction

One of the most serious complications of colonic inflammatory bowel disease (IBD) is colorectal cancer (CRC). Two prevention strategies of CRC are available: regular surveillance colonoscopy with random biopsies and chemoprevention with 5-aminosalicylic acid (5-ASA) treatment. Surveillance colonoscopies are limited strategies during which only 20–50% of colonic neoplasms are detected (1). Epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC. A meta-analysis has estimated that 5-ASA halved the risk of developing CRC or dysplasia in ulcerative colitis (UC) patients and showed a positive correlation between the protection and the treatment duration (2). Also, Eaden *et al*. (3) in a case–control study showed that mesalasine reduced the risk of CRC by 81% in UC patients. Furthermore, functional studies in rodents have shown potent anticarcinogenic effects for 5-ASA in models of sporadic and colitis-associated cancer (4–6).

The mechanisms sustaining the putative antineoplastic property of 5-ASA are still under investigation. However, several *in vitro* studies have demonstrated that the antineoplastic effects of 5-ASA are mediated via inflammatory-dependent and inflammatory-independent mechanisms including the inhibition of nuclear factor-kappaB (7,8), the Wnt/β-catenin pathway (9), regulation of DNA replication checkpoints (10,11) and disruption of tumor growth factor-β pathway (12,13). Another mechanism sustaining the effect of 5-ASA is the induction and activation of peroxisome proliferator-activated receptor-γ (PPARγ) (14). PPARs are nuclear receptors that function as transcription factors regulating the expression of genes involved in cellular differentiation, development, metabolism and tumorigenesis. In the gut, PPARγ is significantly expressed in colonic epithelial cells and exhibits anti-inflammatory and anticarcinogenic effects, notably by interacting with the β-catenin pathway (15–17).

Our laboratory has already demonstrated that PPARγ mediates 5-ASA anti-inflammatory effects in the colon epithelium in mice and in human culture colonic biopsies (15,18,19). The aim of the present study was to test the hypothesis that the antineoplastic effects of 5-ASA were mediated via PPARγ. We studied the antineoplastic effect of 5-ASA first *in vitro* on the proliferation, growth and apoptosis of HT-29 and Caco-2 colon epithelial cell lines and *in vivo* in a mouse model of colon cancer cell xenograft and in azoxymethane (AOM)-induced colon carcinogenesis. The involvement of PPARγ in 5-ASA-induced antineoplastic effect was assessed by the use of PPARγ antagonist GW9662 and the PPARγ knockdown cells (HT-29 ShPPARγ).

Materials and methods

Chemicals

5-ASA, AOM and GW9662 were purchased at Sigma–Aldrich (St Quentin Fallavier, France). Rosiglitazone was ordered at Spi Bio (Massy, France). Etoposide was purchased at (TCI EUROPE N.V., Belgium). For *in vivo* studies in A/JOlaHsd mice, ethyl cellulose granules (Pentasa, Ferring, Switzerland) leading to a 5-ASA ileocolonic release were used.

Cell lines

HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-39) colon carcinoma cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% and 20% fetal calf serum, respectively, antibiotics and 1× essential amino acids for Caco-2 cell line. The construction and validation of the cell line HT-29 PPARγ knockdown (HT-29 ShPPARγ) and its negative control (HT-29 ShLuc) are described in Supplementary Materials and methods, available at *Carcinogenesis* Online.

Assessment of cell growth

HT-29 and Caco-2 cells were treated either with 5-ASA (30 mM) or rosiglitazone (10^{-5} M) with or without GW9662 (10^{-6} M) for 12, 24 or 36 h. Etoposide 50 mM was used as a positive control. These doses of 5-ASA and rosiglitazone were chosen because in previous publications, they have shown to induce an anti-inflammatory effect on cultured cells (15) and are clinically relevant (20,21). Cells were detached with the trypsin/ethylenediaminetetraacetic acid solution before counting. Results were expressed as the mean number of cells counted blindly in four different experiments when a coefficient of variation

Abbreviations: 5-ASA, 5-aminosalicylic acid; AOM, azoxymethane; CRC, colorectal cancer; IBD, inflammatory bowel disease; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor-γ; SCID, severe combined immunodeficiency; UC, ulcerative colitis.

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<10% was obtained. The viability of cells was determined by the trypan blue exclusion test.

Analysis of cell proliferation by Ki-67 immunostaining

After 24 h of culture, HT-29 and Caco-2 cells were treated for 48 h with 5-ASA (30 mM) with or without GW9662 (10^{-6} M). Rosiglitazone (10^{-5} M) treatment was used as control. Cells were fixed in paraformaldehye 4%, permeabilized in phosphate-buffered saline (PBS) containing 0.1% triton X-100 at 4°C and then incubated with 1.5% normal goat normal and blocking buffer (1% bovine serum albumin in PBS). Cell proliferation was assessed by a nuclear Ki-67 staining using a mouse monoclonal primary antibody directed against Ki-67 (dilution 1:50 overnight; ZYMED, Clinisciences, Montrouge, France). An Alexa 594 donkey anti-mouse IgG was used as secondary antibody (dilution 1:100, Molecular Probes, Invitrogen, Cergy Pontoise, France). Nuclei were stained with Hoescht 33342 solution (0.125 mg/ml) (Sigma–Aldrich) and visualized under a fluorescence microscope (Leica, Bensheim, Germany). An irrelevant mouse serum was used as a negative control. At least 500 cells/sample were counted in four different experiments. Results were expressed as the mean ± standard error of the mean percentage of stained cells.

Detection of apoptosis

After 24 h of culture, HT-29 and Caco-2 cells were treated for 24 h with 5-ASA (30 mM) with or without GW9662 (10^{-6} M). Rosiglitazone (10^{-5} M) was used as control ligand of PPARγ and etoposide (50 mM) as an apoptosis inductor. Cells undergoing apoptosis were identified using a TUNEL assay (Roche Diagnostics, Meylan, France). At least 500 cells/sample were counted in four different experiments. Results were expressed as the mean ± standard error of the mean percentage of stained cells.

Xenograft tumor model

Six to seven weeks old pathogen-free BALB/c *SCID* mice (Institut Pasteur, Lille, France) were used. Animals were housed five per cage in barrier environment and had free access to sterile water and food. Human colon cancer cells (10^7 HT-29 cells) pretreated or not with GW9662 for 24 h were implanted subcutaneously in the flank of animals. Two days after cell inoculation, mice were treated with 5-ASA (5 or 50 mM) administered daily by peritumoral injection for 10 or 21 days. The effect of PPARγ during 5-ASA treatment was evaluated by daily intraperitoneal injection of GW9662 (1 mg/kg/day). The control group has received saline instead of 5-ASA. Mice were checked three times a week for tumor development. After killing at 10 or 21 days, tumor size and volume were calculated as described previously using the formula $(L \times W^2)$ 0.5, where L is length and W is width (22). Tumors were weighted before paraffin embedding for histological examination.

Aberrant crypt model

A/JOlaHsd mice have been used in this experiment given the high susceptibility of this genetic background to the carcinogen AOM (23). A/JOlaHsd mice aged of 5 weeks were randomized into four groups each of 10 mice and treated for 7 weeks as follows: Control group fed with standard diet, 5-ASA group fed with 5-ASA ethylcellulose granules-containing chaw at 200 mg/kg/day, 5-ASA+GW9662 group fed with 5-ASA ethylcellulose granules-containing chaw at 200 mg/kg/day and receiving daily intraperitoneal injection of GW9662 at 2 mg/kg/day, GW9662 group fed with standard diet and receiving daily intraperitoneal injection of GW9662 at 2 mg/kg/day. Control and 5-ASA mice groups have received daily intraperitoneal injection of 3% dimethyl sulfoxide/PBS (GW9662 vehicle). One week after 5-ASA and/or GW9662 treatment, all received two intraperitoneal injections of AOM, at 10 mg/kg/day dissolved in isotonic saline, with 1 week interval between doses. At the end of week 7, mice were euthanized and the colon was removed via a midline laparotomy. The colons were opened along the mesenteric border, washed with PBS and pinned flat. The colon was fixed in 4% neutral buffered formalin for 24 h and then washed with PBS. To identify aberrant crypt foci (ACF), colons were stained with 0.2% methylene blue in water for 3 min. The entire mucosal surface was examined using stereomicroscope. ACF were distinguished from normal colonic crypts if they were at least two times greater than normal size, had an irregular crypt cavity and had a densely staining pericryptal zone. The number of ACF per colon and the number of aberrant crypts (ACs) per focus were double blindly recorded (N.E.-J. and L.D.).

Results

5-ASA regulates epithelial cell growth in a PPARγ-dependent manner

HT-29 and Caco-2 cell lines were used to investigate the cellular mechanisms underlying 5-ASA-induced cell growth arrest (Figure 1). To this aim, cells were treated with 5-ASA (30 mM), rosiglitazone

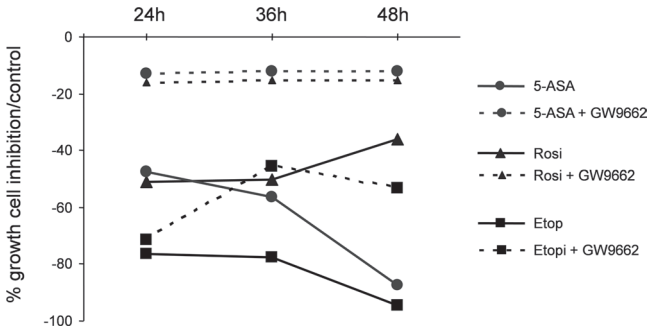

Fig. 1. 5-ASA inhibits cell growth in a PPARγ-dependent way. Incubation of HT-29 cells with 5-ASA (30 mM) resulted in a significant inhibition of cell growth compared with controls. Similar results were obtained in cells treated with the rosiglitazone (rosi, 10^{-5} M) and etoposide (etop, 50 mM). Addition of GW9662 (10^{-6} M) completely abolished the growth inhibitory activities of 5-ASA and rosiglitazone. Results were expressed as the mean number of 500 cells counted blindly in four different experiments.

(10^{-5} M, PPARγ ligand, positive control) and etoposide (50 mM). For instance, etoposide, an anticancer drug belonging to the family of topoisomerase II inhibitors, was used as positive control, which exhibits cytotoxic actions independently of PPARγ. Compared with untreated cells, incubation of HT-29 cells for 24, 36 and 48 h with 5-ASA (30 mM) resulted in a significant inhibition of cell growth to 45, 54 and 85%, respectively ($P < 0.01$). Similar results were obtained in cells treated with the two positive controls, that is, rosiglitazone (10^{-5} M) and etoposide (50 mM) ($P < 0.01$). As expected, GW9662 was not capable of significantly modulating etoposide-induced cell growth inhibition, whereas it abolished this effect on cells treated with 5-ASA or rosiglitazone. Similar results were observed in Caco-2 cells (data not shown).

5-ASA regulates epithelial cell proliferation through a PPARγ-dependent mechanism

To assess the involvement of PPARγ in the potential antiproliferative effect of 5-ASA, we performed immunohistochemistry assays using an antibody directed to the nuclear protein Ki-67. We first verified that HT-29 cells, colonic adenocarcinoma cell lines, were characterized by a high rate of proliferation under basal conditions (94 ± 1% of stained cells). In HT-29 cells, 5-ASA treatment induced a dramatic decrease in the proliferative index (35 ± 4% versus 94 ± 1%, $P < 0.001$). Similar results were obtained in rosiglitazone-treated cells, with less than 10% of cells stained. Furthermore, the proliferation of HT-29 cells was decreased by 95% ($P < 0.0001$) when treated with etoposide (Figure 2A and B). Treatment with GW9662 significantly abolished the antiproliferative effect of 5-ASA and rosiglitazone (38% versus 75%, $P < 0.05$ of stained cells and 10% versus 84%, $P < 0.05$, respectively). The proliferative potential of HT-29 was restored by 40% in cells co-treated with etoposide and GW9662 ($P < 0.05$). Similar results were observed in Caco-2 cells (data not shown).

5-ASA regulates epithelial cell apoptosis through a PPARγ-dependent mechanism

Apoptosis is another major mechanism involved in the regulation of cell growth. In HT-29, no apoptosis was detected by the TUNEL method, which labeled DNA strand breaks. However, etoposide and rosiglitazone induced a high rate of apoptosis (95% and 62%, respectively). As expected, similar results were obtained with 5-ASA treatment (30mM), with 83 ± 1% stained cells (Figure 3A and B). Co-treatment with 5-ASA and GW9662 suppressed the induction of apoptosis (83 ± 1% versus 35 ± 0.1% $P < 0.001$), indicating that 5-ASA proapoptotic effect is at least, in part, dependent of the PPARγ signaling pathway. Similar inhibition of HT-29 apoptosis was obtained in cells co-treated by rosiglitazone and

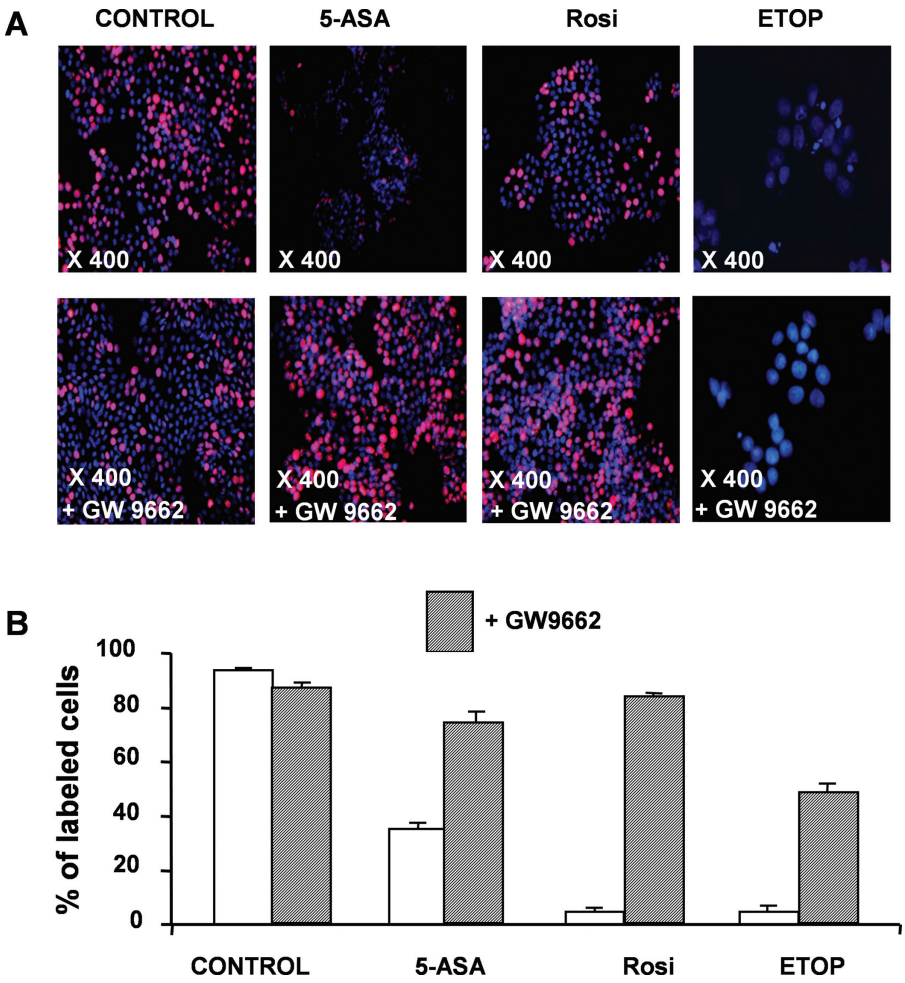

Fig. 2. 5-ASA inhibits proliferation through PPARγ. (**A**) 5-ASA (30 mM) and rosiglitazone (rosi, 10^{-5} M) inhibited HT-29 cell proliferation assessed by the nuclear Ki-67 staining (pink) compared with cells incubated with the medium alone (control). Nuclei were stained in blue with Hoescht 33342 solution. Addition of GW9662 (10^{-6} M) for 24 h blocked the antiproliferative effects of 5-ASA and rosiglitazone. (**B**) In comparison with untreated HT-29 cells, 5-ASA treatment for 48 h inhibited by 63% cell proliferation (35 ± 4% stained cells versus 94 ± 1% stained cells, $P < 0.001$). Co-treatment with GW9662 blocked the antiproliferative effects of 5-ASA. Results expressed as the mean number of 500 cells counted blindly in four different experiments.

GW9662 but not in etoposide-treated cells (Figure 3). Likewise, the proapoptotic effect of 5-ASA in Caco-2 cells (97%) was completely abolished by the addition of GW9662 (3%) (data not shown).

Beside the pharmacological verification of the role of PPARγ in mediating the antineoplastic effects of 5-ASA using GW9662, the effect of 5-ASA was assessed in PPARγ knockdown cells (HT-29 ShPPARγ). Compared with HT-29ShLuc cells, neither 5-ASA (30 mM) nor rosiglitazone (10^{-5} M) had significant effect on the proliferation and apoptosis of HT-29 ShPPAR cells as assessed by Ki-67 immunostaining and TUNEL assay (Supplementary Figure 1B–E, available at *Carcinogenesis* Online).

Taken together, these observations suggest that the regulation of epithelial cell growth, proliferation and apoptosis by 5-ASA are mediated, at least partially, via PPARγ signaling pathway.

5-ASA has an antineoplastic effect in a xenograft tumor model

To evaluate the *in vivo* antineoplasic effect of 5-ASA, severe combined immunodeficiency (SCID) mice engrafted with HT-29 colon cancer cells were treated daily for 21 consecutive days with 5-ASA at 50 mM (Figure 4). At the end of the treatment, a reduction of 80–86% of tumor weight and volume was observed in SCID mice receiving 5-ASA compared with control mice or mice treated with GW9662 alone (Figure 4A–C). The antineoplastic effect of 5-ASA was already detectable after 10 days of 5-ASA treatment. Similar results were obtained with mice treated with 5-ASA at 5 mM (data not shown). Antitumorigenic effect of 5-ASA was completely abolished at 21 days by simultaneous intraperitoneal administration of GW9662. Thus, the observed antineoplastic effect of 5-ASA is at least partially dependent on PPARγ.

Antineoplastic effect of 5-ASA in vivo

In order to examine the direct antineoplastic effect of 5-ASA on mice, a model of AOM carcinogenesis was employed (Figure 5A and B). Mice were treated with 5-ASA and/or GW9662 for 7 weeks as described above. To induce carcinogenesis, mice have received two intraperitoneal injections of AOM at 1 week interval. After 7 weeks, mice were harvested and the formation of ACs and ACF was recorded. A significant decrease in the number of ACs/focus (22%, $P = 0.038$) and ACF/colon (75%, $P = 0.017$) was observed under 5-ASA compared with control mice treated with AOM only (Figure 5C and D). The co-treatment of mice with GW9662 suppressed almost completely the antineoplastic effect of 5-ASA. For instance, mice treated with 5-ASA+GW9662 had increased number of ACF (5.6 ± 0.5) compared with mice treated with 5-ASA only (1.75 ± 0.25), revealing a 68% increase in the number of ACF ($P = 0.04$). Interestingly, GW9662-treated mice had even higher ACF count compared with mice treated with 5-ASA+GW9662 (8.5 ± 0.64 versus 5.6 ± 0.5, $P = 0.003$) and even to control mice (8.5 ± 0.64 versus 7 ± 1.22) (not significant).

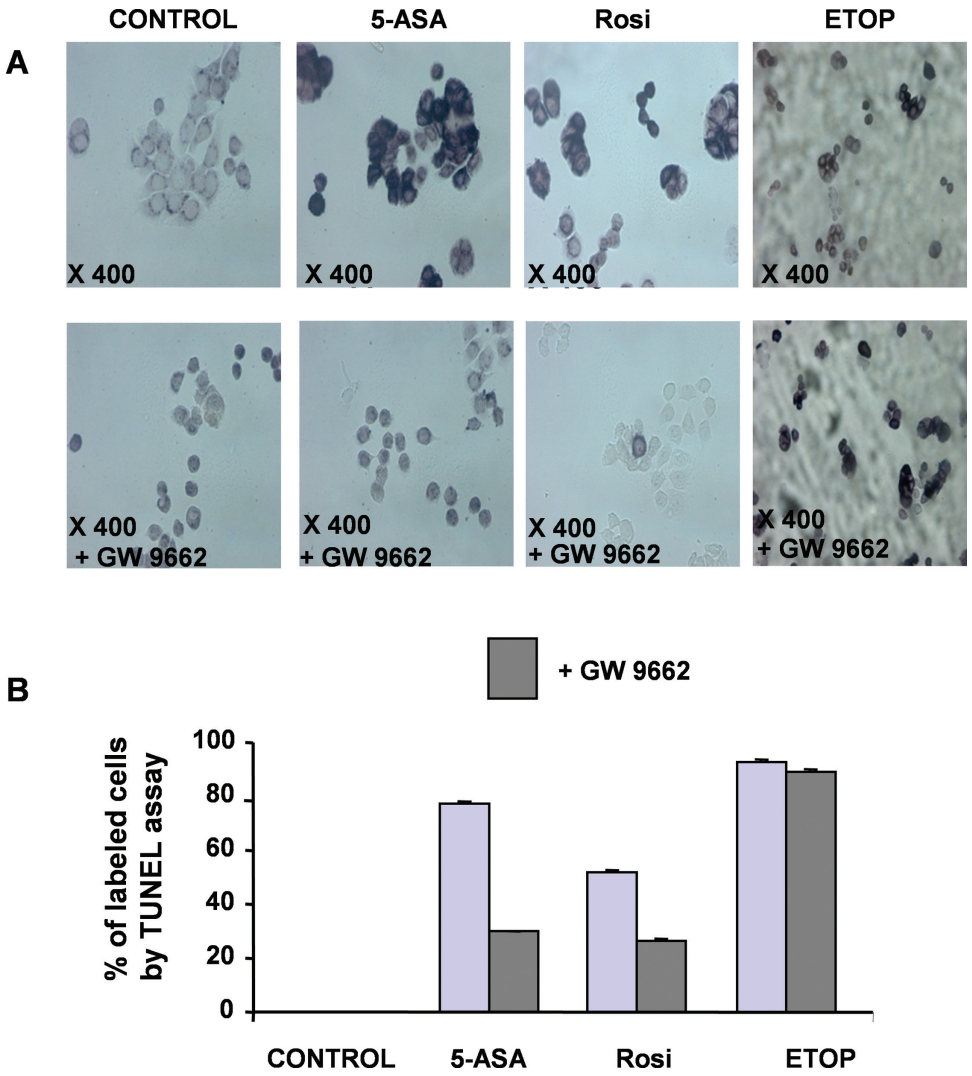

Fig. 3. 5-ASA induces apoptosis through PPARγ. (**A**) Treatment of either 5-ASA (30 mM) or rosiglitazone (10^{-5} M) induced apoptosis measured by TUNEL assay in HT-29 cells. Addition of GW9662 (10^{-6} M) for 24 h completely abolished the proapoptotic effects of 5-ASA and rosiglitazone. (**B**) 5-ASA and rosiglitazone treatment for 48 h induced apoptosis in 83% and 62% of cells, respectively. Co-treatment with GW9662 blocked the proapoptotic effect of 5-ASA and rosiglitazone. Results were expressed as the mean number of 500 cells counted blindly in four different experiments.

Taken together, these data support the antineoplastic properties of 5-ASA *in vivo* and reveal that PPARγ signaling is required for mediating 5-ASA antineoplastic effects.

Discussion

Patients suffering from Crohn's disease and UC displayed an increased risk of developing CRC. Surveillance colonoscopies provided limited protection against CRC due to the technical limitations of detecting dysplastic lesions in normal appearing mucosa (1,24). However, retrospective studies have shown that chemopreventive strategies, employing aminosalicylates, provided enhanced protection for IBD patients against CRC development (25,26). 5-ASA was opted since decades as the treatment of choice of active mild to moderate of IBD, notably UC. In addition to its efficacy and safety as an anti-inflammatory therapy, 5-ASA was also believed to have unique chemopreventive properties. Several studies have shown that regular usage of 5-ASA prevented the development of CRC in IBD patients by decreasing cell proliferation and promoting apoptosis (25,27). However, the mechanisms sustaining 5-ASA chemopreventive properties remain largely unknown. In this work, we demonstrated that the well recognized antineoplastic effects of 5-ASA are mediated via PPARγ. Using clinically relevant concentrations (20,21), we first showed by *in vitro* cell count, Ki-67 immunostaining and TUNEL assay that 5-ASA displayed both antiproliferating and proapoptotic properties. Subsequent to GW9662 application, evidence that PPARγ mediated the chemopreventive effects of 5-ASA was established. Besides, the antiproliferating and proapoptotic properties of 5-ASA were absent in PPARγ knockdown cells that further supports the role of PPARγ. In an *in vivo* model of xenograft tumor, we furthermore showed a strong reduction in tumor development due to topic effect of 5-ASA through a PPARγ-dependent mechanism. Finally, in a model of AOM-induced colon carcinogenesis, 5-ASA significantly suppressed the number of both ACF and ACs via PPARγ signaling pathway.

The antiproliferative and proapoptotic effects of 5-ASA on several tumor-derived cell lines have been previously reported and different mechanisms have been proposed, namely inhibition of nuclear factor-kappaB, prostaglandins (7,8) and cyclo-oxygenase (28); activation of caspase-3 (29–31); inhibition of the Wnt/β-catenin pathway (9); disruption of the epidermal growth factor receptor signaling (13); regulation of DNA replication checkpoints (10,11,31); suppression of oncogenes (32) and disruption of tumor growth factor-β pathway (12,13). However, the receptor mediating these antineoplastic effects of 5-ASA was not revealed. One study has established in wild-type

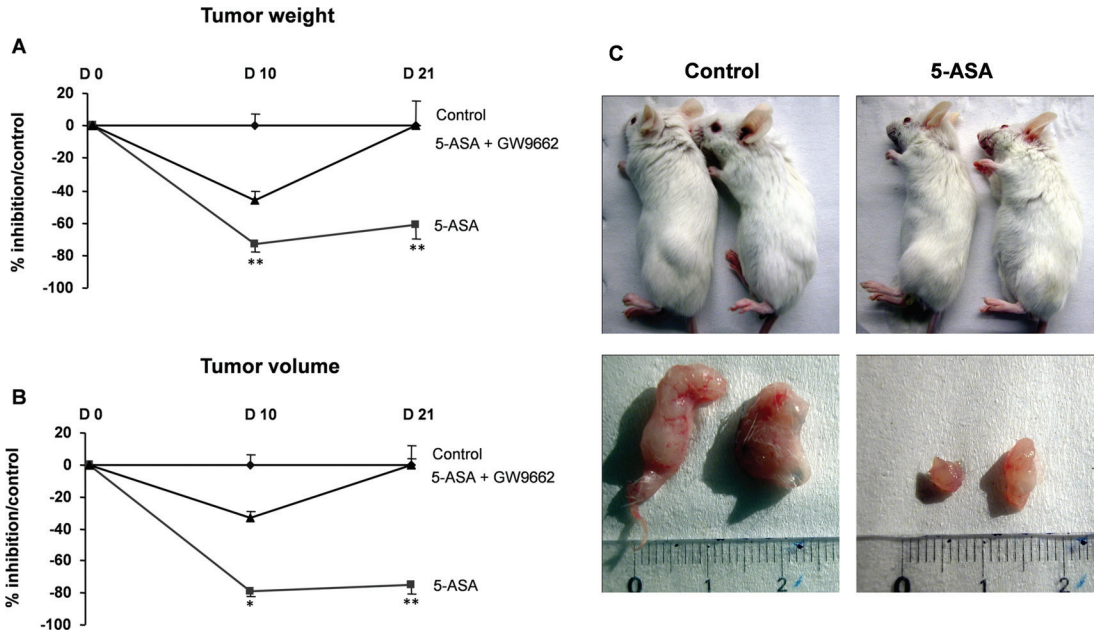

Fig. 4. Xenograft tumor model in SCID mice. 10^7 HT-29 cells pretreated or not pretreated with GW9662 were injected subcutaneously in 6–7-week-old SCID mice. Mice were treated for 21 days at the injection point of HT-29 with 5-ASA (50 mM) or PBS (control). One group of mice was co-treated with a local injection of 5-ASA and an intraperitoneal injection of GW9662 (1 mg/kg/day). Following injection of HT-29 cells, tumor development was monitored at days 10 and 21 and evaluated by tumor weight (**A**) and volume (**B**) measurements. Results are expressed as percentage of inhibition compared with control mice. Topical subcutaneous treatment of 5-ASA (50 mM) reduced the tumor weight by 60% and volume by 83% compared with controls after 10 days of treatment. Injection of PPARγ antagonist GW9662 (1 mg/kg/day) significantly abolished the inhibition of tumor growth induced by 5-ASA. (**C**) Illustrative photo of the tumor mass in control and 5-ASA groups taken upon animal harvest and showing the effect of 5-ASA on tumor size. *$P < 0.05$ for D10 compared to D0; **$P < 0.05$ for D21 compared to D0.

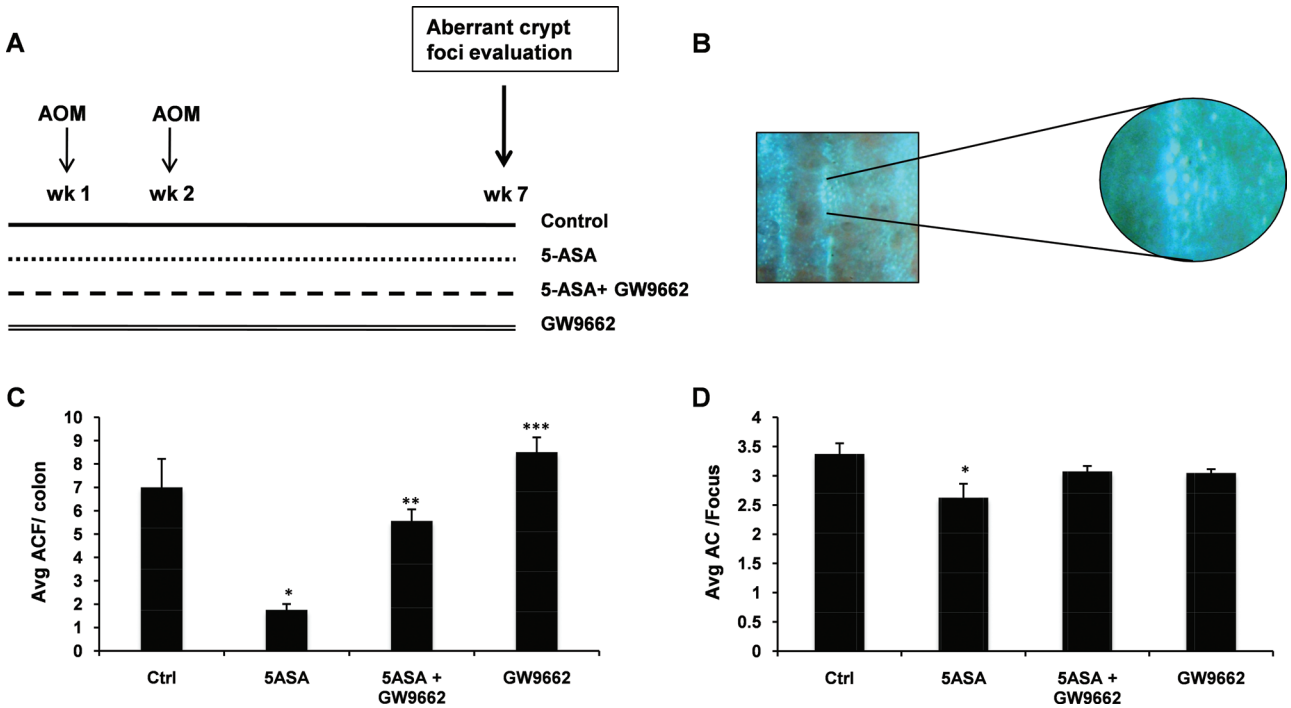

Fig. 5. 5-ASA exhibits a potent anticarcinogenic effect *in vivo*. (**A**) A/JOlaHsd mice were randomly divided into four groups: Control group (Ctrl) fed with standard diet, 5-ASA group fed with 5-ASA granules-containing chaw at 200 mg/kg/day, 5-ASA+GW9662 group fed with 5-ASA granules-containing chaw at 200 mg/kg/day and receiving daily intraperitoneal injection of GW9662 at 2 mg/kg/day, GW9662 group fed with standard diet and receiving daily intraperitoneal injection of GW9662 at 2 mg/kg/day. Control and 5-ASA mice groups have received daily intraperitoneal injection of 3% dimethyl sulfoxide/PBS (GW9662 vehicle). To induce ACs (**B**), mice have received intraperitoneally two doses of AOM (10 mg/kg/day) at weeks 2 and 3. At the end of the protocol (week 7), mice were harvested and the formation of ACs and ACF was blindly recorded. (**C** and **D**) 5-ASA treatment induced a significant reduction of ACF (75%) and ACs (22%) compared with mice fed with standard diet. Coadministration of GW9662 suppressed the antitumoral effect of 5-ASA. GW9662-treated group displayed higher number of ACF compared with 5-ASA+GW9662 group. *$P < 0.05$ for 5ASA compared to control; **$P < 0.05$ for 5ASA + GW9662 compared to 5ASA; ***$P < 0.05$ for GW9662 compared to 5ASA + GW9662.

and PPARγ-negative CRC cell lines treated with 5-ASA that PPARγ was involved in 5-ASA-mediated induction of apoptosis. In this work, Schwab *et al*. (31) showed that mesalazine regulates cell cycle and caspase activity in a PPARγ-dependent and PPARγ-independent pathways in colonocytes. However, this study was limited to *in vitro* experiments and even showed mesalazine antiproliferative effects in dominant-negative PPARγ cells. In our work, we further established, thanks to the specific effect of GW9662 and to PPARγ knockdown cells, that the antiproliferative and proapoptotic actions of 5-ASA were at least partially PPARγ dependent. Regarding the *in vivo* antineoplastic effects of 5-ASA, data from rodent models of CRC were contradictory. Several studies have shown that using chemical CRC animal models, 5-ASA and other PPARγ ligands reduced the number of ACF, as well as the rate of tumor cell proliferation, and increased tumor apoptosis (4–6,17,33). On the other side, in APC mouse model of multiple intestinal neoplasia not only 5-ASA was found without any antineoplastic properties (34) but also PPARγ ligands were shown to enhance colon carcinogenesis (17,33,35,36). A possible explanation for such discrepancies is that colon carcinogenesis in APC^{Min} mice was driven by germ-line mutations in the *Apc* gene. It has been shown that mice with preexisting damage to *APC*, a regulator of β-catenin, develop tumors in a manner insensitive to the status of PPARγ. Thus, PPARγ can suppress colon carcinogenesis only before damage to the APC/β-catenin pathway (37). In this work, the results are in good agreement with previous reports showing antitumorigenic role for 5-ASA, but we demonstrated for the first time the link between the role of 5-ASA in suppressing colon carcinogenesis and its signaling through PPARγ. Interestingly, blockage of PPARγ with GW9662 induced ACF formation in the colon of mice reflecting the important endogenous antineoplastic role of PPARγ (Figure 5).

Several clinical studies have evaluated the potential chemopreventive effect of PPARγ ligands, including 5-ASA. For example, in patients with chemotherapy-resistant metastatic CRC, troglitazone treatment did not induce any objective tumor response and all patients had progressive disease as their best response to therapy (38). In fact, troglitazone, as all thiazolidinediones, has a wide systemic distribution due to broad expression of PPARγ. This might explain from one side its inefficiency in tumor prevention and highlights and from the other side the need for further development of ligands targeting tumor cells. What adds a further level of complexity is that UC patients were shown to have impaired colonic expression of PPARγ, which might also reduce drug efficiency (39). In this perspective, 5-ASA galenic presentations of delayed release have been developed so that to act locally in the colon and to be absorbed by colonic epithelial cells. Therefore, the efficiency of this drug is related to its mucosal concentration and systemic dosages remain low after oral or rectal administration (20). Thus, the availability of 5-ASA at the cellular level could explain its superior efficacy in preventing CRC among IBD subjects compared with other PPARγ ligands. Notably, Rubin *et al.* (26) observed a 3.4-fold risk reduction of dysplasia and CRC in UC patients treated with ≥1.2 g/day of 5-ASA. In a case–control study comparing 102 patients with UC and CRC to matched controls, it was shown that CRC risk was reduced by 75% under 5-ASA regular treatment compared with no 5-ASA use (40). Likewise, a nested case–control study involving 18 969 patients with IBD in the UK General Practice Research Database (1987–2001) showed that regular 5-ASA users had a significantly reduced risk of CRC (adjusted odds ratio 0.6) compared with irregular 5-ASA users (41). On the other hand, some retrospective studies did not support 5-ASA as protective in preventing CRC in IBD patients (42–44) potentially due to the heterogeneity of the populations analyzed and the short period of exposure to 5-ASA. Furthermore, because of the design of retrospective studies, some key areas have not been sufficiently addressed such as concurrent use of other medications and accurate data on dosing.

In our experimental model, 5-ASA treatment (200 mg/kg/day) was started 1 week before the injection of the carcinogenic AOM and maintained for 6 weeks afterward. The treatment consisted of 5-ASA granules, usually used in IBD therapy, administered orally in mouse chaw in order to mimic tablet medication in humans. Whether this protocol reflects the chemoprevention strategy in patients with IBD is arguable. In case–control studies, regular users, having decreased risk of CRC, were defined as having at least 6 months of non-interrupted 5-ASA treatment at doses ranging between 1.2 and 2 g/day (26,40,41). Nevertheless, the accurate dosage and duration of 5-ASA pharmacological therapy targeting CRC prevention are not yet well established. Moreover, little information is available regarding the potential chemopreventive effect of other IBD medications, and it is currently unclear whether a patient would benefit from enhanced chemoprevention when combining, for example, 5-ASA and another treatment (e.g., azathioprine). Finally, despite its well-characterized antineoplastic activities, it remains to be determined the step at which 5-ASA would interfere in the carcinogenesis process. Although there is complete absence of data from humans, studies in rodent models of colon cancer have shown that 5-ASA was able to prevent tumor formation and further to reduce the number of developed tumors (5,6,34). Although these observations warrant further investigation since they were detected in various animal models, using different dosages and administration routes, they may reveal that 5-ASA would act at multiple stages of the carcinogenesis.

5-ASA was originally developed without any knowledge of its molecular targets. However, in the past years, our knowledge of its role as a chemopreventive agent in IBD-related CRC has increased. Our piece of work, establishing the key role of PPARγ in mediating 5-ASA antineoplastic prevention, may lead to the development of more effective PPARγ ligands with higher efficiency or for use in association with agents of additive or synergic effects.

Supplementary material

Supplementary Materials and methods and Figure 1 can be found at http://carcin.oxfordjournals.org/

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