

# Mesalamine Suppresses the Expression of TC22, a Novel Tropomyosin Isoform Associated with Colonic Neoplasia

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## ABSTRACT

Although a protective role for mesalamine against colon cancer in ulcerative colitis has been shown epidemiologically, its molecular mechanism is unknown. We cloned and sequenced a novel human tropomyosin (hTM) isoform, TC22, which is an alternatively spliced variant of normal epithelial hTM isoform 5 (hTM5), identical apart from 25 C-terminal amino acids. TC22 is expressed in 100% of colorectal carcinoma but is not expressed in normal colon epithelial cells. To explore a molecular mechanism of chemoprevention, we examined the effect of mesalamine on TC22 expression using LS180 colon cancer cells. Expression of hTM5 and TC22 was investigated at the protein and gene levels by fluorescence-activated cell sorting and real-time reverse transcription-polymerase chain reaction. Small interference RNA (siRNA) against the TC22 variant were transfected into LS180 colon cancer cells, reducing protein and transcript levels by 45 to 50%. Mesalamine or sulfasalazine (2

mM), but not sulfapyridine, significantly ( $p < 0.02$ – $0.006$ ) reduced the expression of the TC22 transcript and significantly ( $p < 0.05$  to  $<0.0002$ ) reduced the expression of TC22 protein in a dose-dependent and reversible manner. Rosiglitazone, a specific peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) agonist, similarly and significantly ( $p < 0.002$ ) reduced TC22 protein expression. A polymerase chain reaction array of 84 cancer-related genes performed on TC22 siRNA-transfected cells demonstrated a significant (more than two times) change in targets involved in apoptosis, adhesion, angiogenesis, and tissue remodeling. We conclude that mesalamine, sulfasalazine, and rosiglitazone significantly reduced the cellular expression of TC22, implicating PPAR $\gamma$  in this modulation. Similar suppression of TC22 by siRNA produced gene level changes on several critical carcinogenic pathways. These findings suggest a novel antineoplastic molecular effect of mesalamine.

Patients with ulcerative colitis (UC) have an increased risk of developing colorectal cancer (CRC), ranging from 2% within the first decade, 8% in the second, and 18% after 30 years of disease (Ekbom et al., 1990; Eaden et al., 2001). Sulfasalazine (SASP) and its active metabolite 5-Aminosalicylate (5-ASA, mesalamine) have been the first-tier drugs of choice in the management of UC. Epidemiological studies suggest that SASP (Moody et al., 1996) and 5-ASA (Rubin et al., 2007) may reduce the risk of CRC in UC, with an odds ratio of 0.51 at lower doses and 0.23 at dosages greater than 1.2 g/day (Velayos et al., 2005). The mechanism of this anti-

carcinogenic effect of SASP and 5-ASA remains unknown (Croog et al., 2003).

Despite several decades of research, the mode of action of 5-ASA in UC is unclear. Various mechanisms suggested include altering the bacterial flora, antioxidant effects, and modulating the immune functions (Harris et al., 1972; West et al., 1974; Rubinstein et al., 1978; Shanahan et al., 1986). Although 5-ASA is a weak cyclooxygenase (COX) inhibitor, it is an effective lipoxygenase inhibitor, suppressing LTB<sub>4</sub> and sulfido-peptide release, which is more clinically relevant in UC (Sharon et al., 1978; Peskar et al., 1987). 5-ASA has also been shown to reduce the tumor size of CRC in a rat model (Davis et al., 1992). In both in vivo and in vitro experiments, it was implicated in inducing apoptosis, reversibly reducing cell proliferation (Reinacher-Schick et al., 2000, 2003). The appeal of 5-ASA as a cancer-preventing medication is understandable because azobonded sulfasalazine (SASP) bypasses

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**ABBREVIATIONS:** UC, ulcerative colitis; 5-ASA, mesalamine, 5-aminosalicylate; COX, cyclooxygenase; CRC, colorectal cancer; PPAR, peroxisome proliferator-activated receptor; SASP, sulfasalazine; siRNA, small interference RNA; SP, sulfapyridine; FACS, fluorescence-activated cell sorting; TNF, tumor necrosis factor; TZD, thiazolidinedione; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; TM, tropomyosin; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aa, amino acid(s); MMP, matrix metalloproteinase; PDGFA, platelet-derived growth factor A; VEGFA, vascular endothelial growth factor A; IGF1, insulin-like growth factor 1; ITGA, integrin  $\alpha$ ; OD, optical density; mAb, monoclonal antibody.

small-bowel absorption to be cleaved by the colonic bacterial azoreductase enzyme, allowing the 5-ASA moiety to achieve very high intraluminal concentrations in the colon. Of the two metabolites of SASP, 5-ASA and sulfapyridine (SP), 5-ASA acts topically on the colonic mucosa and is largely excreted in the stool, whereas SP is absorbed from the colon and is excreted mainly in the urine (Das et al., 1973; Das and Dubin, 1976; Frieri et al., 1999).

It has been suggested that the effect of 5-ASA may actually be mediated via peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Rousseaux et al., 2005). Viewed from a physiological standpoint, PPARs are activated by fatty acids and transduce metabolic signals into transcriptional responses via specific nuclear response elements. PPAR $\gamma$  is expressed in several cell types, most highly in adipocytes and colonic epithelium, and it is essential for mucosal integrity (Dubuquoy et al., 2002; Wu, 2003). PPAR $\gamma$  heterodimerizes in the nucleus with retinoid X receptor  $\alpha$ , and this complex binds to DNA response elements, which increase nuclear factor- $\kappa$ B, c-Jun, and c-fos as well as decrease mucosal inflammatory cytokines, such as interleukin-1 $\beta$ , TNF- $\alpha$ , and several chemokines (Su et al., 1999; Dubuquoy et al., 2002). Studies have also suggested a role in the use of PPAR $\gamma$  agonists in the treatment of patients with inflammatory bowel disease (Su et al., 1999; Lewis et al., 2001, 2008; Dubuquoy et al., 2003) and in the prophylaxis of UC-related neoplasia in animal models (Tanaka et al., 2001; Girnun and Spiegelman, 2003; Osawa et al., 2003). Trinitrobenzene sulfonic acid colitis model using PPAR $\gamma$ (+/-) mice was found to be refractory to 5-ASA, whereas wild-type mice largely responded. Likewise, *in vitro* studies showed that 5-ASA paralleled the actions of rosiglitazone, a commercially available thiazolidinedione (TZD), in the induction of PPAR $\gamma$  mRNA, nuclear migration, and protein expression in HT29 cells (Rousseaux et al., 2005).

In UC, we reported autoimmune responses (both humoral and cellular) against human tropomyosin isoform 5 (hTM5), a cytoskeletal microfilament protein that is predominantly expressed in normal colonic epithelium (Geng et al., 1998; Taniguchi et al., 2001). More recently, using the cDNA library from the colon cancer cell line T84, we have cloned and sequenced a novel hTM isoform, TC22, which is strongly associated with colonic neoplasia and carcinoma with 100% sensitivity but is not detected in normal colon epithelium (Lin et al., 2002). TC22 is identical to hTM5 apart from the C-terminal domain, representing amino acids 222 to 247 coding exon 9. TC22 is an alternatively spliced variant of hTM5 that is expressed during the neoplastic process (Lin et al., 2002). A study of nondysplastic, inflammatory colonic tissues from 65 patients with UC showed that TC22 expression was significantly associated with disease extent and duration (Geng et al., 2007), two conditions strongly associated with carcinogenesis in UC. This suggests that TC22 expression may be an important biomarker for the identification of patients with UC who are at high risk for colon cancer and provides the possibility of its modulation as a tool for exploring chemoprevention.

In this study, we describe the effects of SASP and its metabolite, 5-ASA, on the cellular expression of TC22 in the human colon cancer cell line LS180 at both the protein and gene expression levels. The effect of 5-ASA treatment on TC22 is characterized in terms of dose-response, reversibility, and the effect of retreatment. To evaluate the specificity

of the effect of 5-ASA on TC22, we also examined the effect of the SASP, SP, acetylsalicylic acid (aspirin), a potent nonsteroidal anti-inflammatory agent (sulindac), and a commercially available PPAR $\gamma$  agonist (rosiglitazone). We also explored the possibility of these drugs inducing apoptosis in this colon cancer cell line model. Finally, to investigate the potential pathophysiological significance of TC22 modulation on carcinogenesis, we suppressed TC22 expression via specific siRNA and studied the effect on an array of cancer-related targets.

## Materials and Methods

**Cell Culture.** LS-180 colon cancer cells (American Type Culture Collection, Manassas, VA) were grown in DMEM with 10% fetal bovine serum. SASP, 5-ASA, sulfapyridine, aspirin, and sulindac were obtained from Sigma-Aldrich (St. Louis, MO). Various concentrations of drugs were made fresh in serum-free DMEM. For SP and SASP, the medium was first made alkaline to allow the dissolution of the compounds and then pH-adjusted to pH 7.3. Solutions of both 5-ASA and SASP tend to precipitate at pH 7.4 at higher molarities. Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI), dissolved in DMSO, and used at a concentration of 10  $\mu$ M (Rousseaux et al., 2005; Han and Roman, 2006). A DMSO-treated group was included as a vehicle control in all experiments that involved rosiglitazone.

The intraluminal concentrations of 5-ASA in patients with inflammatory bowel disease receiving SASP or mesalamine maintenance therapy have been reported as approximately 7.0 to 14.0 mM (Lauritsen et al., 1984; Allgayer, 2003). In a series of initial experiments using 0 to 20 mM 5-ASA, we have observed that 5-ASA at the molarity of 10 mM or greater is toxic to cells in culture. Thus, after a series of titration experiments, a more conservative final concentration of 1 to 2 mM 5-ASA was used for all of the subsequent experiments. As an additional control, we also used 2 mM aspirin in parallel. Sulindac (1 mM) was chosen based on established literature (Shiff et al., 1995).

Cells were incubated with 10 ml of serum-free DMEM, with and without the drugs. To examine the "reversibility" or "irreversibility" of the effect seen with 5-ASA, cells were incubated for 4 h with the drug, washed three times, and incubated for 24 h either with serum-free DMEM or retreated with the respective drug solution. After these daily reincubations for up to 5 days, cells were removed with 0.25% Trypsin-EDTA, counted, and stained with trypan blue to verify that >90% were viable in every experiment. Cells were washed, frozen in part for Western blot analysis, or fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA) for 20 min at 4°C for FACS analysis.

**Western Blot and ELISA Analyses Using Specific Monoclonal Antibodies.** We have described previously the development of specific monoclonal antibodies against hTM5 (hTM5 IgM) and TC22 (TC22-4 IgG) (Geng et al., 1998; Lin et al., 2002). The specificity and sensitivity of these antibodies to various human tropomyosin (TM) isoforms synthesized recombinantly was verified by Western blot and by ELISA methods as reported previously (Lin et al., 2002). The reactivity was detected by chemiluminescence method.

**FACS Analysis.** Cells were suspended with the following primary antibodies: CG3 IgM, 1  $\mu$ l (1:100) for hTM5; TC22-4 IgG, 1  $\mu$ l (1:100) for TC22; and unrelated isotype control monoclonal antibodies (MOPC IgG or IgM) in equal concentrations. After incubation at 4°C overnight, cells were washed with 30 volumes of phosphate-buffered saline/0.5% bovine serum albumin/2 mM EDTA twice. Cells were resuspended and incubated at 4°C for 1 h with secondary antibodies, cyanine-2 conjugate G- $\alpha$ -M IgM or IgG, washed, and hTM5 and TC22 expression was detected by fluorescent-activated cell sorting within 1 h, measuring emission at 506 nm.

**Real-Time RT-PCR Assay.** RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. The RNase-Free DNase set (Qiagen) was used during RNA purification. cDNA was generated using Advantage RT-for-PCR kit (Clontech, Mountain View, CA). DNA contamination was tested by PCR of the reverse-transcriptase samples. All runs were accompanied by a negative control, which included all reagents except for cDNA. Expression of target mRNA was normalized with respect to actin using the  $\Delta\Delta C_t$  method.

Real-time RT-PCR was performed by two independent methods for the TC22 transcript (GenBank accession number AY004867) and by one method for the hTM5 transcript (GenBank accession numbers X04588, BC000771, and AK026559). SYBR Green detection was completed for both TC22 and hTM5 transcripts on the LightCycler (Roche, Indianapolis, IN) using the Qiagen SYBR Green PCR kit. All samples underwent 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 20 s, and extending at 72°C for 20 s. Primers for TC22 were 5'-CTG AGT TTG CTG AGA GAT CGG TAG-3' and 5'-AGG TCA GTG GTG TGA GCA GTA AG-3'. Primers for hTM5 were 5'-GAT AAA CTC AAG GAG GCA GAG ACC-3' and 5'-GAC TGG GCG TTC TAC ATC TCA T-3'.

In addition, TaqMan assays were completed for TC22 in parallel with the SYBR assays. TaqMan analysis was performed with the Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan Universal PCR Master Mix. All samples underwent 40 cycles of denaturing at 95°C for 10 s and annealing/ extending at 60°C for 1 min. TaqMan Gene Expression Assay (assay ID Hs01080278\_mH) was commercially obtained from Applied Biosystems for analysis of the TC22 gene product, which included forward and reverse primers and the oligonucleotide probe.

**siRNA against TC22.** Silencer select predesigned siRNA against the TC22 gene product (GenBank accession number AY004867) were commercially obtained from Ambion (Austin, TX). Transfection was performed on  $0.1 \times 10^6$  cells/well using siPORT transfection reagent (Ambion). Titration experiments identified an optimum concentration of 30 nM siRNA to effectively silence TC22 protein and gene products. Cells were transfected in parallel with CY3-labeled GAPDH to assess transfection efficiency and scrambled siRNA as a negative control. Medium was changed 24 h after transfection, and cells were observed for CY3 fluorescence. Cells were harvested at 48 h, and transfection efficiency was also assayed at this time using GAPDH antibody.

**PCR Assay of Carcinogenic Pathways.** RT<sup>2</sup> Profiler Human Cancer Pathway Finder PCR Array was obtained from SABiosciences (Frederick, MD). Untransfected, scrambled siRNA (negative control), and TC22-siRNA-transfected LS180 cells were assayed via an optimized, real-time RT-PCR reaction for 84 genes known to be involved in human carcinogenesis according to the manufacturer's protocol.

**Apoptosis.** Apoptosis was examined by Vybrant Apoptosis Assay Kit 2 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

**Statistical Analysis.** Statistical analysis was performed by a one-tailed, paired, Student's *t* test against the null hypothesis of no significant change. All average values are displayed with error bars indicating S.E.M.

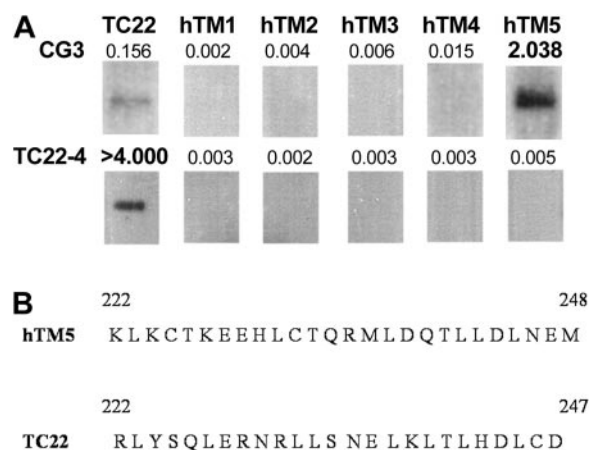
## Results

**Specificity and Sensitivity of the Monoclonal Antibodies against hTM5 and TC22.** Using recombinant isoforms of hTM (hTM1–hTM5) and TC22, we determined the sensitivity and specificity of CG3 and TC22-4 by ELISA and Western blot assays (Fig. 1A). CG3 monoclonal antibody (mAb) reacts with the N-terminal residues (aa 4–10) of hTM5, which are common to both hTM5 and TC22. However, CG3 reacts with hTM5 with much greater intensity than

with equimolar concentrations of TC22, as indicated by the OD values (top of each lane, Fig. 1A) and by the visualized bands. Because hTM5 is a structural, major cytoskeletal protein in colonic epithelial cells (Geng et al., 1998) and TC22 is an alternatively spliced variant, hTM5 is far more abundant in the cells (Lin et al., 2002). Therefore, at cellular concentrations of TC22 and hTM5, the cross-reactivity of CG3 to TC22 is likely to be of minimal significance. The TC22-4 antibody is both highly sensitive and specific to the unique C-terminal region of the TC22 peptide (aa residues 222–247) that distinguishes it from hTM5 (Fig. 1B). All ELISA OD values against hTMs 1, 2, 3, 4, and 5 by TC22-4 were negative; however, it strongly reacted both by ELISA (OD >4.000) and Western blot against the recombinant TC22 protein (Fig. 1A).

**Specific Reduction of TC22 Protein by 5-ASA.** LS180 colon cancer cells were incubated with 2 mM 5-ASA for 0.5, 1, 1.5, 2, 3, 4, 8, and 24 h. The viability of the cells was greater than 90% after each incubation. Figure 2, A and B, shows the expression of TC22 and hTM5 at various time points after incubation with 2 mM 5-ASA. There was no significant change in TC22 expression at 0.5 or 1 h (Fig. 2A). However, at 1.5 h, TC22 expression decreased sharply on average by 45.03% (S.E.M.  $\pm$  1.66,  $p$  < 0.0117) compared with the 0 h time point and continued to decrease by 49.17% (S.E.M.  $\pm$  7.28,  $p$  < 0.0468) at 2 h, by 38.03% (S.E.M.  $\pm$  5.78,  $p$  < 0.0341) at 3 h, by 49.10% (S.E.M.  $\pm$  7.03,  $p$  < 0.0002) at 4 h, by 45.60% (S.E.M.  $\pm$  3.87,  $p$  < 0.027) at 8 h, and by 49.39% (S.E.M.  $\pm$  5.89,  $p$  < 0.0002) at 24 h. Therefore, a significant 40 to 50% reduction of TC22 expression was observed starting within 1.5 h of treating the cells with 2 mM 5-ASA and was sustained for 24 h without further addition of the drug (Fig. 2, A and B). It is important to note that the suppression of TC22 was specific, because as hTM5 expression examined in parallel remained essentially unchanged (Fig. 2A).

This effect on TC22 was found to be dose-dependent, be-



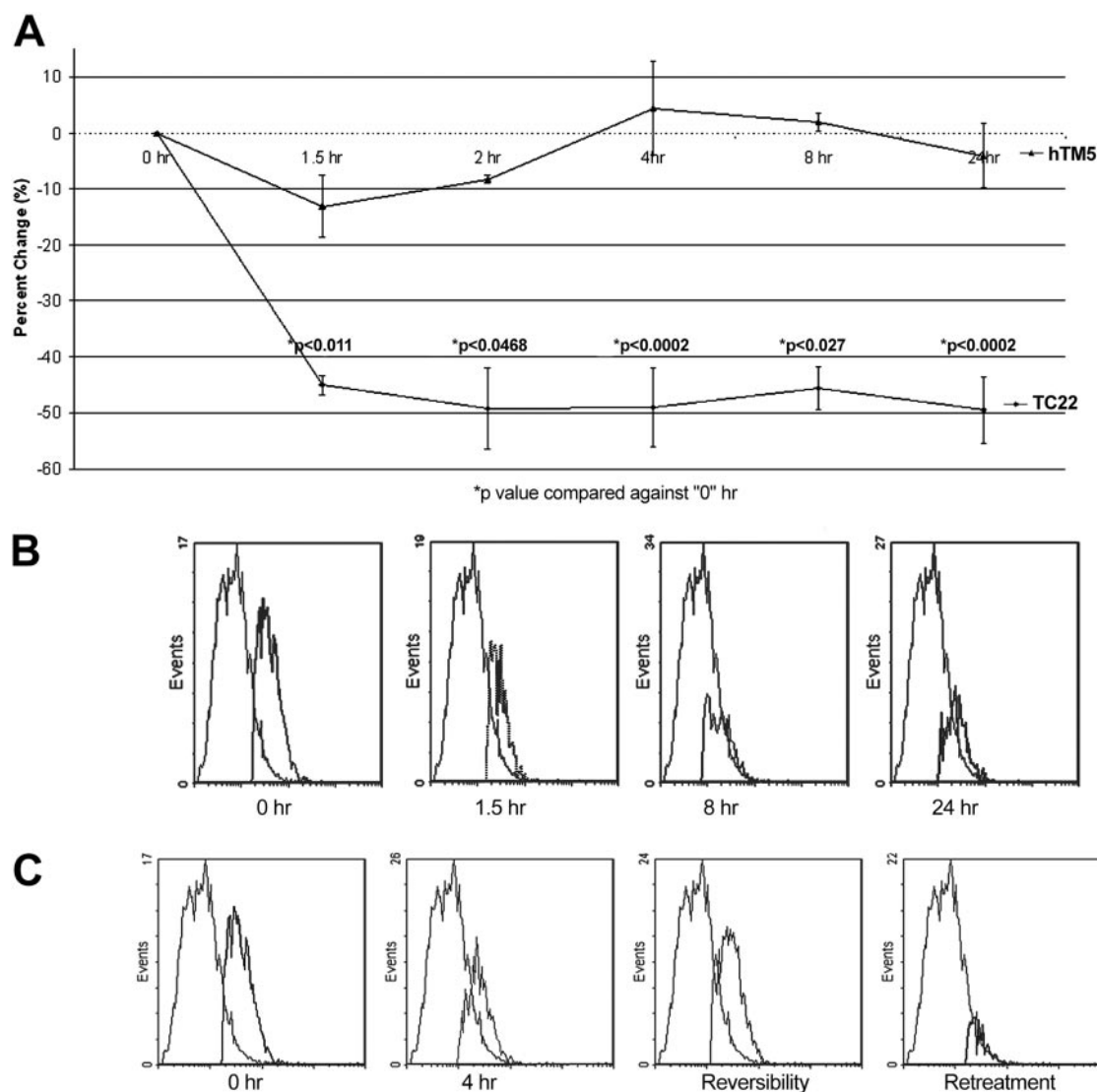
**Fig. 1.** A, reactivity of TC22-4 and CG3 antibodies to hTM isoforms by ELISA and Western blot. Western blot analysis of anti-hTM5 (CG3) and anti-TC22 (TC22-4) mAbs against recombinant hTM isoforms hTMs 1 to 5 and TC22 (50 ng/lane). Corresponding ELISA values are shown above each lane. The specificity of the respective mAbs is evident. Because the epitope recognized by CG3 mAb is against aa residues 4 to 10 of hTM5, which is common in both hTM5 and TC22, CG3 activity against TC22 can also be seen. However, TC22-4 mAb raised against the C-terminal peptide of TC22, as shown in B, did not react with hTM5. B, amino acid sequence for hTM5 and TC22. The sequence is identical for both proteins from amino acid residues 1 to 221. However, at the C-terminal domain, aa residues 222 to 247 are different.



cause 1 mM 5-ASA treatment reduced TC22 by only 26.91% (S.E.M.  $\pm$  8.79) at 2 h of treatment. However, 2 mM 5-ASA at the same 2-h time point reduced TC22 expression by 49.17% ( $p < 0.0468$ ) (Fig. 3A). We also analyzed whether the effect of 2 mM 5-ASA on TC22 expression was reversible if 5-ASA exposure was removed and whether retreatment with a fresh batch of drug caused any further effect (Fig. 2C displays a representative experiment). When the LS180 cells were incubated with 2 mM 5-ASA for 4 h, washed, replaced with 10 ml of serum-free DMEM, and harvested after 24 h, the TC22 suppression was only 11.35% (S.E.M.  $\pm$  2.68,  $p < 0.0737$ ) compared with pretreatment value. However if the LS180 cells were incubated with 2 mM 5-ASA for 4 h, washed, and retreated with fresh 2 mM 5-ASA solution, the suppression was 59.02% (S.E.M.  $\pm$  6.336,  $p < 0.0057$ ), greater on average than the suppression seen with single treatments at 4

(49.10%) or 24 h (49.39%). Cells were also retreated daily with fresh solutions of 2 mM 5-ASA for up to 5 days, showing a continued suppression of TC22 by 32.96% (S.E.M.  $\pm$  9.07,  $p < 0.012$ ) at 72 h and by 46.09% (S.E.M.  $\pm$  6.74,  $p < 0.001$ ) at 5 days. Therefore, the effect of 5-ASA on TC22 expression was observed to be dose-dependent, reversible, and enhanced with continued treatment.

**Effect of Other Drugs on Expression of TC22.** Several related compounds were also investigated for their effect on TC22 expression and hTM5 expression in vitro (Fig. 3B). At 4 h, we found that 2 mM SASP significantly reduced TC22 expression by 44.4% (S.E.M.  $\pm$  16.52,  $p < 0.03$ ) compared with the pretreatment value, whereas 2 mM SP had no effect. Rosiglitazone (10  $\mu$ M) also significantly reduced TC22 expression by 41.0% (S.E.M.  $\pm$  8.3,  $p < 0.002$ ), similar to the level of modulation by 5-ASA and SASP. Neither SASP, SP,



**Fig. 2.** A, summary of FACS data using 2 mM 5-ASA treatment on TC22 and hTM5 expression in LS180 cells. A significant inhibition of TC22 (40–50%) is noted at 1.5 h that is sustained over 24 h. However, hTM5 levels remain essentially unchanged ( $\pm 10\%$ ). Each data point is derived from  $n = 3$  to 10 experiments, mean  $\pm$  S.E.M. B, effect of 2 mM 5-ASA on TC22 expression in LS180 cells at various time points. A representative example of FACS data of TC22 expression after exposure to 5-ASA (2 mM) is shown. The first peak is the profile when the cells were incubated with unrelated mAb, MOPC IgG. The second peak is the FACS profile of TC22 reactivity at various time intervals after subtraction of MOPC IgG profile by the Overton method. C, reversibility and the effect of retreatment with 2 mM 5-ASA on TC22 expression in LS180 cells. The suppression of TC22 is reversible after removal of the drug at 4 h and incubation with serum-free medium for 24 h (Reversibility). However, retreatment of the cells at 4 h with a fresh solution of 5-ASA (2 mM) showed further suppression of TC22 expression (Retreatment).

nor rosiglitazone affected cellular expression of hTM5 (data not shown). The large S.E.M. of SASP is due to the difficulty in keeping SASP in solution at pH 7.2 to 7.4. However, the suppression was still significant ( $p < 0.03$ ) compared with the pretreatment value (Fig. 3B).

We also treated the cells with 2 mM aspirin because of its structural similarity with 5-ASA. In contrast to 5-ASA, 2 mM aspirin decreased TC22 only by 7.31% (S.E.M.  $\pm$  3.8), which was not significantly different from the pretreatment value. Sulindac (1 mM) decreased TC22 by only 13.9% (S.E.M.  $\pm$  5.57,  $p < 0.070$ ) (Fig. 3B). Thus, neither sulindac nor aspirin significantly affected cellular TC22 or hTM5 expression.

**Apoptosis.** At 4 h of treatment with 5-ASA or SASP, there was no significant change in apoptosis noted (Fig. 4). Although these values were not statistically significant, there was a numerical increase in the cell populations of early apoptotic cells in the FACS analysis (Fig. 4). There was no notable difference in necrotic cells or late apoptotic cells among the groups, suggesting low toxicity of the drug at the concentrations used.

**Real-Time RT-PCR Assay.** Real-time RT-PCR assays were conducted in parallel with the protein experiments at 4 and 24 h of incubation with 5-ASA. These experiments demonstrated a significant, 50 to 60% suppression of the TC22 transcript at 4 h, which was verified both by SYBR ( $p < 0.006$ ) and TaqMan ( $p < 0.018$ ) assays (Fig. 5). At 24 h, the transcript level for TC22 returned to the baseline. hTM5 transcription measured on the same samples by the SYBR method showed no significant change at both 4 and 24 h (Fig. 5).

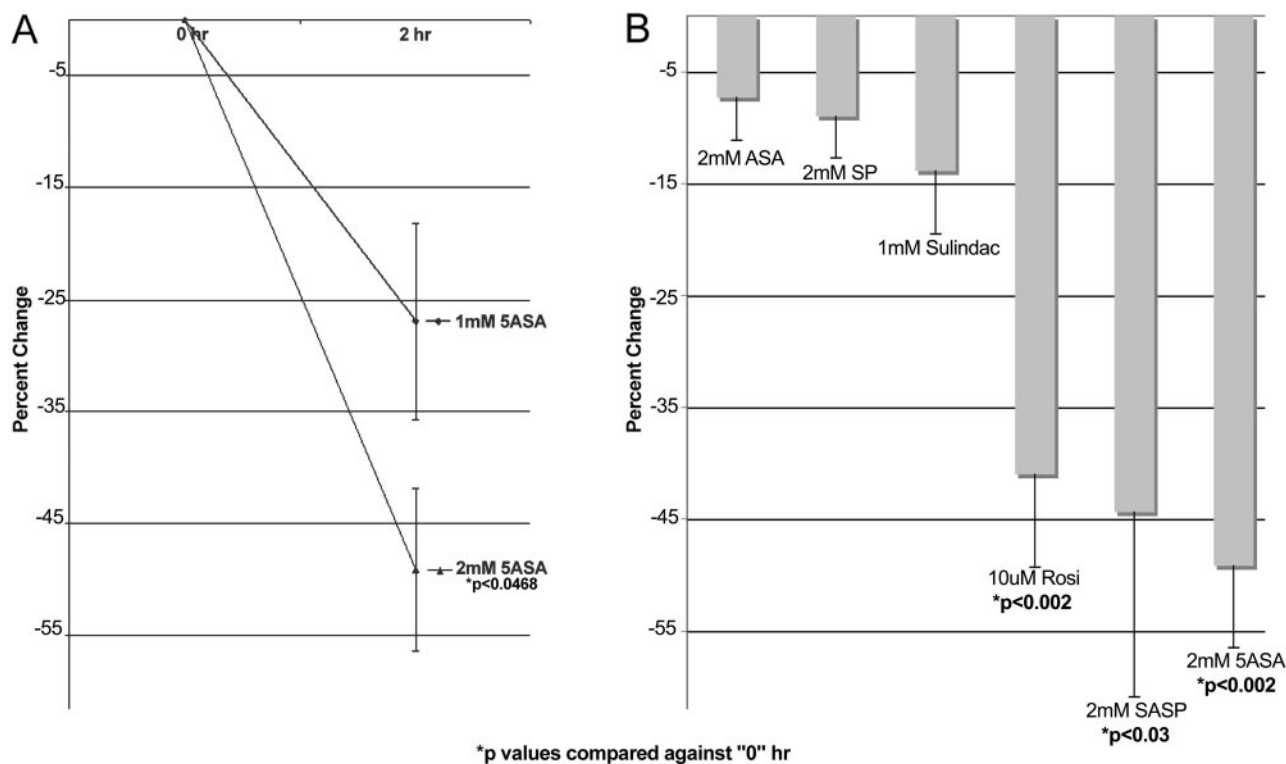
**TC22 Silencing by siRNA and Carcinogenesis PCR Array.** LS180 colon cancer cells were transfected with TC22

siRNA in parallel with CY-3-labeled GAPDH siRNA and were assessed for efficiency at 48 h (Fig. 6A). Success of the transfection was verified at the protein level by FACS, demonstrating a 53% decrease in TC22 protein expression in the transfected cells compared with a 2% change in TC22 protein expression in the scrambled siRNA-negative control cells (Fig. 6B). RT-PCR demonstrated a similar (45%) decrease in TC22 transcript levels in the TC22 siRNA-transfected cells.

Examination of TC22 siRNA-transfected cells by an 84-gene focused carcinogenic array (RT<sup>2</sup> Profiler Human Cancer Pathway Finder PCR Array; SABiosciences), compared with untransfected cells, generated the modulations shown in Table 1. Similar changes were observed comparing the data from the transfected cells with those from the scrambled siRNA negative control. Most notably, among the statistically significant ( $>2$ -fold) changes observed, TC22 silencing was predominantly proapoptotic, with increases of two TNF superfamily products [TNFRSF10B (+3.1-fold), TNFRSF25 (+2.0-fold)] and involved in cell adhesion with increases in two integrin subunits [ITGA1 (+2.6-fold), ITGA3 (+3.0-fold)]. In addition, the array indicates that suppression of TC22 may induce angiogenesis [IGF1 (+6.9-fold), TEK (+12.7-fold), VEGFA (+3.1-fold), PDGFA (−2.0-fold)] and two matrix metalloproteinases [MMP1 (+9.2-fold) and MMP2 (+3.6-fold)].

## Discussion

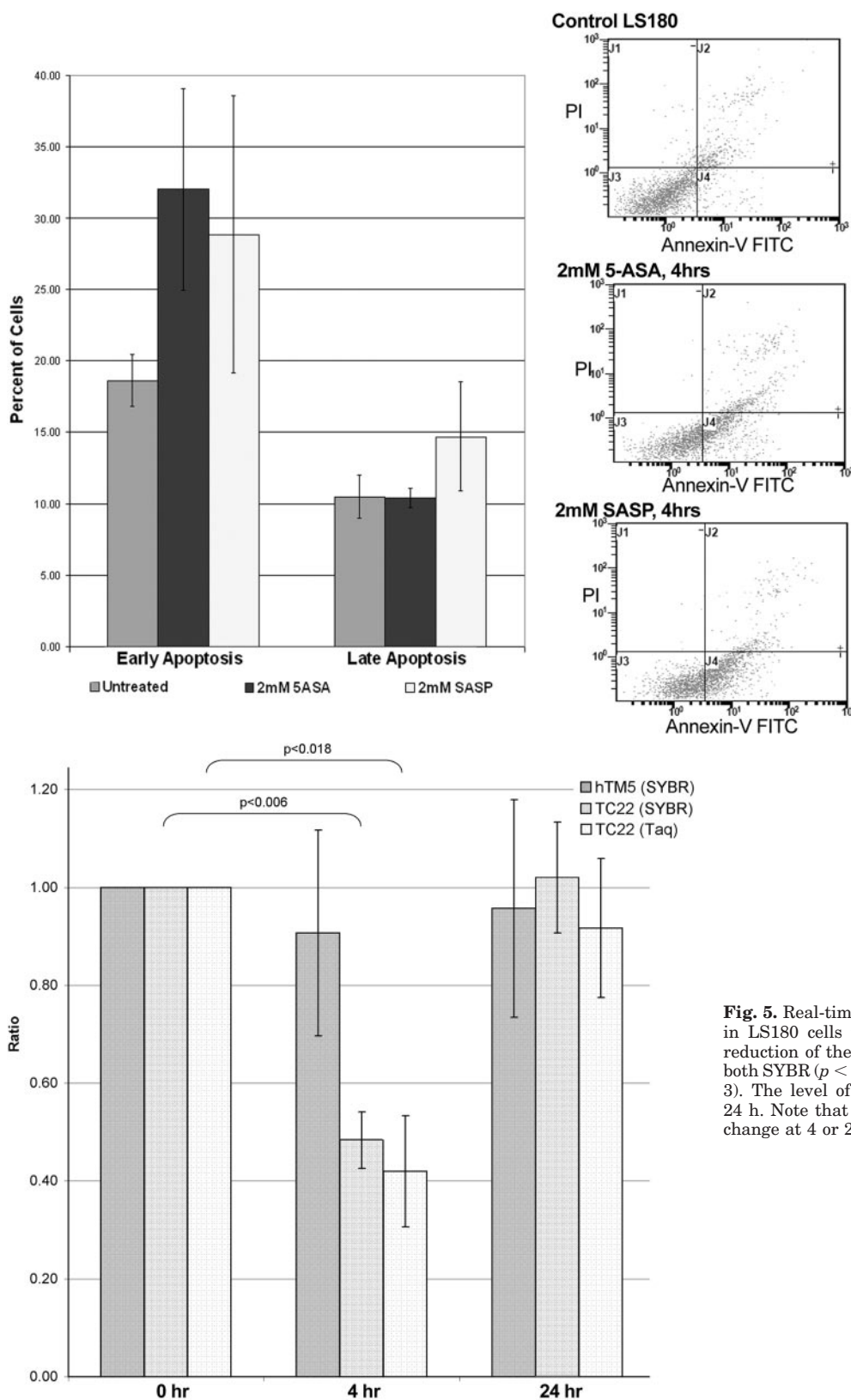
Our study shows that 5-ASA and SASP specifically decrease the cellular expression of TC22, a novel biomarker of colon cancer. The inactive metabolite of SASP, SP, failed



**Fig. 3.** A, dose-response of 5-ASA on TC22. Dose-response of 1 and 2 mM 5-ASA on TC22 expression at 2 h. 5-ASA (2 mM) was found to be the appropriate concentration for suppression of TC22 expression ( $n = 3$ ). B, effect of various drugs on TC22 expression in LS180 cells at 4 h. TC22 expression in LS180 cells at 4 h of treatment with aspirin (2 mM), SP (2 mM), sulindac (1 mM), rosiglitazone (Rosi) (10  $\mu$ M), sulfasalazine (SASP) (2 mM), and 5-ASA (2 mM) by FACS. Significant suppression was evident with 5-ASA, SASP, and rosiglitazone but was not found with aspirin, SP, and sulindac ( $n = 3$  experiments).

to produce any effect on TC22. A concentration of 2 mM in vitro was chosen because of measured intraluminal concentrations of 7.0 to 14.0 mM 5-ASA in patients with inflammatory bowel disease receiving maintenance therapy (Lauritsen et al., 1984; Allgayer, 2003), titration ex-

periments indicating cell toxicity in culture greater than 10 mM, and experimental difficulties in the solubility of SASP at pH 7.4. The specific reduction in cellular expression of TC22 and not the normal colon epithelial hTM isoform, hTM5, by 5-ASA and SASP is particularly intriguing.



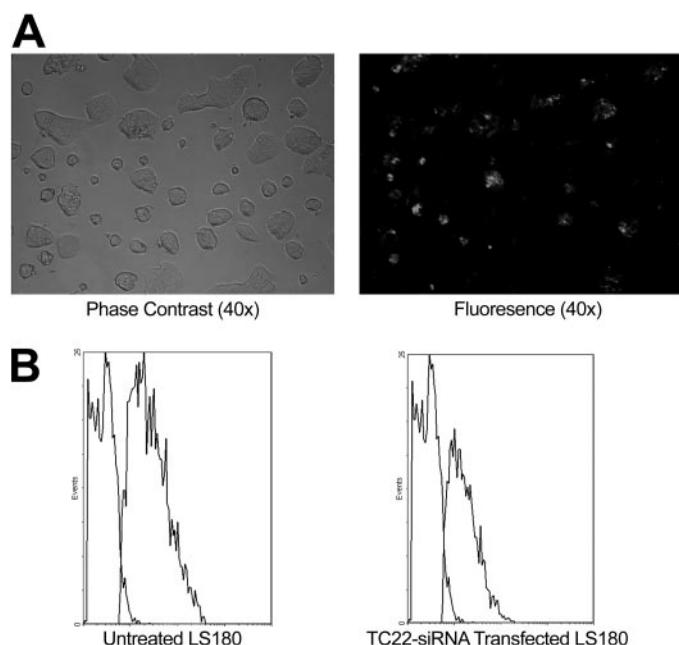
**Fig. 4.** Early and late apoptosis upon exposure of LS180 cells to 2 mM 5-ASA or 2 mM SASP for 4 h. Left, mean value ( $\pm$  S.E.M.) of early apoptosis and late apoptosis from three experiments. Right, FACS profile demonstrating a rightward shift toward greater propidium iodide and Annexin V positivity. No statistically significant differences in apoptosis were noted compared with untreated cells. FITC, fluorescein isothiocyanate.

**Fig. 5.** Real-time RT-PCR for hTM5 and TC22 transcripts in LS180 cells treated with 2 mM 5-ASA. A significant reduction of the TC22 transcript at 4 h was measured by both SYBR ( $p < 0.006$ ) and TaqMan ( $p < 0.018$ ) assays ( $n = 3$ ). The level of TC22 transcript returned to baseline at 24 h. Note that the hTM5 transcript did not significantly change at 4 or 24 h.

ing because TC22 is strongly associated with colonic neoplasia and carcinoma but is not detected in normal colon epithelium (Lin et al., 2002). We reported previously that 21 of 22 colon cancer specimens showed TC22 expression, whereas only 1 of 17 noncancer colonic mucosal samples and 0 of 13 hyperplastic polyps expressed the protein ( $p < 0.0001$ ) (Lin et al., 2002). Although the specific physiologic and pathophysiologic roles of TC22 remain unclear, this association with the carcinogenic process is further strengthened by the data we present here demonstrating the modulation of several cancer-related pathways after silencing the TC22 gene product. Of particular note is the induction of two subtypes of the TNF superfamily, both

because of the clinical relevance of the modulation of TNF molecules in UC and because of the potentially proapoptotic effect of these specific receptors in TC22-suppressed cells. In addition, several other pathways seem to be affected by the specific loss of TC22, including notable increases in VEGFA and several other genes involved in angiogenesis. However, as demonstrated by our experiments on apoptosis, these genetic modulations by treatment with mesalamine or silencing of the TC22 gene product were not fully realized into functional changes in tumor cell behavior. Although others have shown 5-ASA to induce apoptosis in vitro at concentrations in excess of 40 mM (Bus et al., 1999), our results suggest that at a clinically relevant dosage of 2 mM, there was no significant increase in apoptosis. Although our data failed to demonstrate statistical significance, we did observe increased populations of cells in early apoptosis in individual experiments, suggesting that at higher drug concentrations, this finding may become significant. Thus, although we were able to establish a firm link between the suppression of TC22 by 5-ASA with a variety of relevant genetic targets related to carcinogenesis, our data did not show significance in biological endpoints, such as apoptosis, at these concentrations. Further study is clearly warranted to elucidate the physiologic effects of TC22 modulation.

We studied the level of transcription of TC22 by both SYBR and TaqMan assays at 4 h of treatment because the suppression of the TC22 protein by FACS was most consistently observed at this time point. The suppression of the TC22 transcript is up to 50 to 60% ( $p < 0.018$  to  $< 0.006$ ) at 4 h; however, unlike the protein level, it returns to baseline by 24 h. It is possible that although the suppression of the TC22 protein by 5-ASA was sustained for 24 h, the effect on the gene is shorter in duration. Accordingly, an increase in protein amount might be observed beyond 24 h because the effect of the drug weakens with time, and the transcript level returns to normal within the cell. This hypothesis is further supported by the data showing that the protein suppression by the drug was both reversible and increased with retreatment. These dose-response data also suggest the importance of the clinical use of higher concentrations of 5-ASA,  $>1.2$  g/day, which were shown epidemiologically to be particularly chemopre-



**Fig. 6.** Silencing of TC22 by siRNA. A, efficiency of siRNA Transfection. Cy3-TC22 siRNA-transfected LS180 cells seen by phase contrast and fluorescence (magnification, 45 $\times$ ) 48 h after transfection. B, suppression of TC22 protein. A representative example of FACS data of TC22 expression in the untransfected and TC22 siRNA-transfected cells. Decrease of TC22 protein by 53% was noted in the transfected cells compared with the untransfected control. The first peak is the profile when the cells were incubated with the unrelated mAb, MOPC IgG. The second peak is the FACS profile of TC22 reactivity, scaled, after subtraction of MOPC IgG profile by the Overton method.

**TABLE 1**  
Effect of TC22-siRNA on gene level changes related to carcinogenesis

	Gene	Description	Change
			-fold
Apoptosis signaling	<i>TNFRSF10B</i>	TNF receptor superfamily containing intracellular death domain that transduces apoptosis.	+3.1
	<i>TNFRSF25</i>	TNF receptor superfamily, possibly involved in regulating lymphocyte homeostasis. Stimulates nuclear factor- $\kappa$ B activity and regulates cell apoptosis.	+2.0
Cell adhesion	<i>ITGA1</i>	Integrin, $\alpha$ 1	+2.6
	<i>ITGA3</i>	Integrin, $\alpha$ 3 (CD49C)	+3.0
Angiogenesis	<i>IGF1</i>	Insulin-like growth factor 1/somatomedin C	+6.9
	<i>TEK</i>	TEK tyrosine kinase (related to TIE family) found in endothelial cells interacts with angiopoietin-1 to mediate endothelial cell-smooth muscle cell communication in venous morphogenesis.	+12.7
Matrix metalloproteinases	<i>VEGFA</i>	Vascular endothelial growth factor A	+3.1
	<i>PDGFA</i>	Platelet-derived growth factor A	-2.0
	<i>MMP1</i>	Breakdown of extracellular matrix in embryonic development and	+9.2
	<i>MMP2</i>	reproductive tissue remodeling.	+3.6



ventative (Velayos et al., 2005; Rubin et al., 2007). The reversibility of TC22 expression after the removal of 5-ASA in vitro and the enhancement of suppression after retreatment may provide experimental evidence for the importance of clinical compliance (Kane et al., 2001) in the reduction of dysplasia/colorectal cancer in UC (Moody et al., 1996; Croog et al., 2003; Velayos et al., 2005; Rubin et al., 2007).

SASP contains approximately one-third 5-ASA and two-thirds SP by weight. Therefore, at presumably the "same" experimental concentration of 2 mM, SASP significantly ( $p < 0.03$ ) reduced TC22 by a similar amount as three times the dosage, by weight, of 5-ASA (49.10% for 5-ASA versus 44.4% for SASP). Thus, the suppression of TC22 by SASP seems to be unique, in that it cannot be explained exclusively because of the effect of the 5-ASA moiety present in SASP alone. The data raise the possibility that had we normalized our experimental condition not by molarity, but rather by the total amount of 5-ASA present, there may have been an even greater suppression of TC22 by SASP than that with 5-ASA alone. However, such a dosage of SASP would in vivo cause a significant adverse effect because of the SP moiety (Das et al., 1973) and would in vitro prove to be cytotoxic and difficult to dissolve at pH 7.4.

It has been suggested that the antineoplastic effect of 5-ASA in UC may be due to its continuous anti-inflammatory effects, which may protect against colorectal cancer. However, other more potent anti-inflammatory agents, such as corticosteroids and immunomodulators, such as 6-mercaptopurine (Matula et al., 2005), were unable to show similar antineoplastic effects in epidemiological settings. Because of some structural similarity between 5-ASA and aspirin, the mode of action of 5-ASA is often confused with that of aspirin, and their unique antineoplastic effects assumed to be caused by a similar mechanism.

In actuality, there are considerable differences biochemically and pharmacologically between these two drugs with regard to physical structure and pharmacokinetics (Fig. 7). There are also notable differences in terms of availability to the tissue, primarily systemic for aspirin versus primarily topical for 5-ASA, as well as mode of action, primarily COX-mediated for aspirin versus essentially COX-unrelated for 5-ASA. The biological functions of the two compounds also differ considerably. Whereas aspirin has well demonstrated thrombolytic effects, 5-ASA does not. It is important to note that the suppression of TC22 observed here is specific to 5-ASA and its parent drug SASP and is not observed in an identical dosage of aspirin and a potent inhibitor of the COX pathway, sulindac. Taken together, these data suggest that the reduction in TC22 expression is via a COX-independent mechanism.

It has been demonstrated that the anti-inflammatory effect of 5-ASA may be mediated in part via the PPAR $\gamma$  pathway (Rousseaux et al., 2005). In human trials of TZDs, beneficial effects have been reported in mild to moderate UC with up to 44% response and 17% remission (Lewis et al., 2001, 2008; Girnun and Spiegelman, 2003). Our results indicate that rosiglitazone, a TZD and specific agonist of PPAR $\gamma$ , reduced the expression of TC22 by almost the same amount (41.0%,  $p < 0.002$ ) as 5-ASA (49.2%) and SASP (44.4%) within 4 h. These data provide evidence that the antineoplastic effect of 5-ASA may be mediated via the induction of the PPAR $\gamma$  pathway.

Thus, the specific reduction of TC22 expression, a biomarker for colon carcinogenesis, by SASP, 5-ASA, and the PPAR $\gamma$  agonist rosiglitazone may provide a novel molecular mechanism for mesalamine's epidemiologically observed anticancer potential in patients with UC.

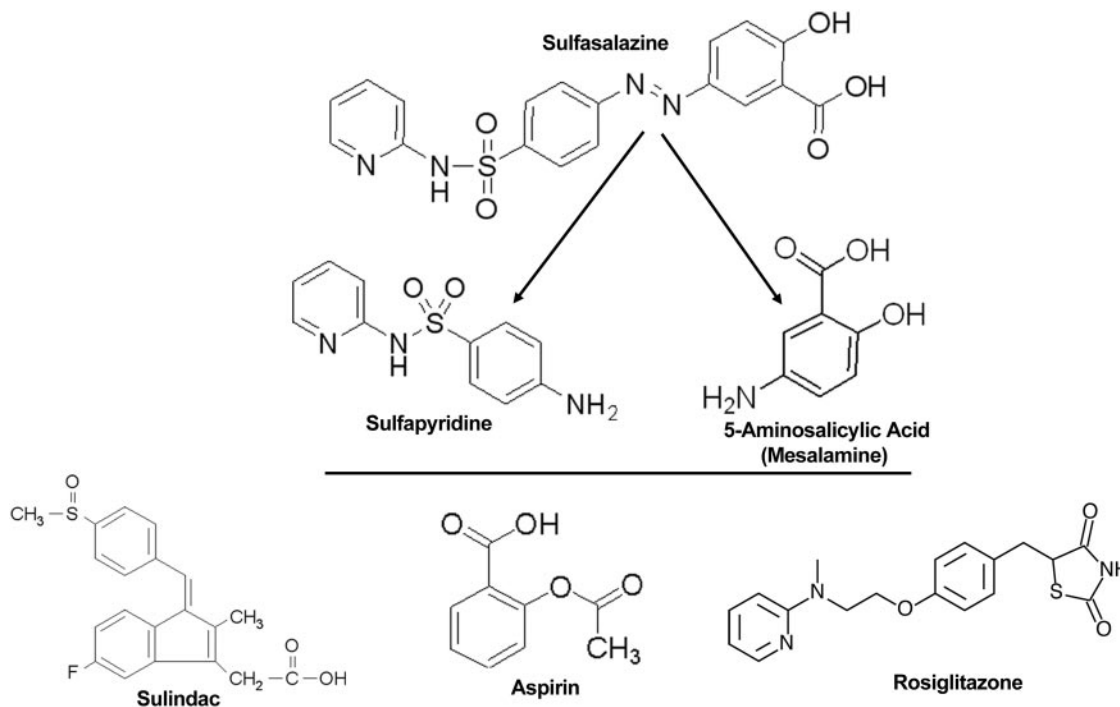


Fig. 7. Structures of the various compounds used.



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