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

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a)   **Running Title Page:** Mesalamine and anti-carcinogenesis in ulcerative colitis

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c)   Number of text pages: 17

Number of tables: 1

Number of figures: 7

Number of references: 39

Number of words in Abstract: 252

Number of words in Introduction: 905

Number of words in Discussion: 1186

d)   Abbreviations: 5-ASA, mesalamine or 5-aminosalicylate; COX, cyclooxygenase;

CRC, colorectal cancer; PPARγ, peroxisome proliferators-activated receptor γ;

SASP, sulfasalazine; siRNA, small interference RNA; SP, sulfapyridine; TNBS,

trinitrobenzene sulfonic acid; UC, ulcerative colitis

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Abstract

Background: Though 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 not expressed by 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. **Methods:** Expression of hTM5 and TC22 was investigated at the protein and gene levels by FACS and real time RT-PCR. siRNA against the TC22 variant were transfected into LS180 colon cancer cells, reducing protein and transcript levels by 45-50%. **Results:** Two mM of mesalamine or sulfasalazine, 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-γ (PPARγ) agonist, similarly and significantly ($p<0.002$) reduced TC22 protein expression. A PCR Array of 84 Cancer related genes performed on TC22 siRNA transfected cells demonstrated a significant ($>2x$) change in targets involved in apoptosis, adhesion, angiogenesis, and tissue remodeling. **Conclusion:** Mesalamine, sulfasalazine, and rosiglitazone significantly reduced the cellular expression of TC22, implicating PPARγ in this modulation. Similar suppression of TC22 by siRNA, produced gene level changes on several critical carcinogenic pathways. These findings suggest a novel anti-neoplastic molecular effect by 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) as well as 5-ASA (Rubin et al., 2003) 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 1.2g/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, anti-oxidant effects, and modulating the immune functions (West et al., 1974, Harris et al., 1972, Rubenstein et al., 1978; Shanahan et al., 1986). Though 5ASA is a weak cyclooxygenase (COX) inhibitor, it is an effective lipoxygenase inhibitor, suppressing LTB4 and sulfidopeptide-CT release, which is more clinically relevant in UC (Sharon et al., 1978; Peskar et al., 1987). 5-ASA has also been shown to reduce 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; Reinacher-Schick et al., 2003). The appeal of 5-ASA as a cancer preventing medication is understandable as azobonded sulfasalazine (SASP) bypasses small bowel absorption to be cleaved by the colonic bacterial azo-reductase 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 mainly excreted in the urine (Das et al., 1973, Das and Dubin, 1976; Frieri et al., 1999).

Recently it has been suggested that the effect of 5-ASA may actually be mediated via Peroxisome Proliferator-Activated Receptor γ (PPARγ) (Rousseaux et al, 2005). Physiologically, PPARs are activated by fatty acids and transduce metabolic signals into transcriptional responses via specific nuclear response elements. PPARγ is expressed in several cell types, with highest levels in adipocytes and colonic epithelium and it is essential for mucosal integrity (Wu, 2003; Dubuquoy et al., 2002). PPARγ heterodimerizes in the nucleus with retinoid X receptor alpha and this complex binds to DNA response elements which increase NFκB, c-jun, c-fos, NFAT and decrease mucosal inflammatory cytokines like IL-1β, TNF-α, and chemokines (Dubuquoy et al., 2002; Su et al., 1999). Studies have also suggested a role in the use of PPARγ agonists in the treatment of inflammatory bowel disease patients (Su et al., 1999, Lewis et al., 2001; Dubuquoy et al., 2003; Lewis et al., 2008) and in the prophylaxis of UC related neoplasia in animal models (Tanaka et al., 2001, Girnun and Spiegelman, 2003; Osawa et al., 2003). TNBS colitis model using PPARγ +/- mice was found to be refractory to 5ASA whereas wild type mice largely responded. Similarly, *in vitro* studies showed that 5ASA paralleled the actions of rosiglitazone, a commercially available Thiazolidinedione (TZD), in the induction of PPARγ 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, which 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 caricinoma 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 222-247 coding exon 9. TC22 is an alternatively spliced variant of hTM5 that is expressed during the neoplastic process (Lin et al., 2002). Recently, a small study of non-dysplastic, inflammatory colonic tissues from patients with UC showed that TC22 expression was associated with UC complicated by primary sclerosing cholangitis and long standing pancolitis (Geng et al., 2004), the two conditions strongly associated with carcinogenesis in UC. This suggests that TC22 expression may be an important biomarker for the identification of UC patients 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 both at 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 re-treatment. To evaluate the specificity of the effect of 5-ASA on TC22, we examined the effect of the SASP, SP, acetyl salicylic acid (aspirin), a potent non-steroidal anti-inflammatory agent (sulindac), as well as a commercially available PPARγ agonist (rosiglitazone). We also explored the possibility of these drugs inducing apoptosis in this colon cancer cell line model. Finally, to explore the potential pathophysiologic 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 (ATCC, Rockville, MD) were grown in DMEM with 10% fetal bovine serum. SASP (S0883), 5-ASA (A3537), Sulfapyradine (S6252), Aspirin (A5376), and Sulindac (S8139) were obtained from Sigma-Aldrich, USA. Various concentrations of drugs were made fresh in serum free DMEM medium. For SP and SASP, the medium was first made alkaline to allow dissolution of the compounds and then pH adjusted to pH 7.3. Solutions of both 5ASA and SASP tend to precipitate at pH 7.4 at higher molarities. Rosiglitazone (71740) was obtained from Cayman Chemical (Ann Arbor, MI), dissolved in DMSO, and used at concentration of 10μM (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 IBD patients receiving SASP or mesalamine maintenance therapy have been reported as approximately 7.0-14.0 mM (Lauritsen et al., 1984; Allgayer, 2003). In a series of initial experiments using 0-20 mM 5-ASA, we have observed that 5-ASA at the molarity of 10 mM or above is toxic to cells in culture. Thus after a series of titration experiments a more conservative final concentration of 1mM to 2mM 5-ASA was used for all of the subsequent experiments. As an additional control we also used 2mM aspirin in parallel. One mM Sulindac was chosen based on established literature (Shiff et al., 1995).

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Cells were incubated with 10mL 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 hrs with the drug, washed three times and incubated for 24 hours either with serum free DMEM or re-treated with the respective drug solution. After these daily re-incubations 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 Diego, CA) for 20min at 4°C for FACS analysis.

Western Blot & ELISA Analyses Using Specific Monoclonal Antibodies

We have previously described 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 earlier (Lin et al., 2002). The reactivity was detected by chemiluminescence method.

FACS Analysis

Cells were suspended with the following primary antibodies: CG3 IgM 1 μL (1:100) for hTM5, TC22-4 IgG 1 μL (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 vol. of PBS/0.5% BSA/2mm EDTA twice. Cells were resuspended and incubated at 4°C for 1 hour with secondary antibodies, Cy2

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(Cyanine)-conj. G-α-M IgM or IgG, washed, and hTM5 and TC22 expression was detected by florescent activated cell sorter within 1 hr, measuring emission at 506nm.

Real Time RT-PCR Assay

RNA was extracted using the *Qiagen RNeasy Mini Kit* per manufacturer's instruction. The RNase-Free DNase set (Qiagen) was used during RNA purification. cDNA was generated using *Advantage RT-for-PCR kit*. DNA contamination was tested by PCR of the RT samples. All runs were accompanied by a negative control which included all reagents, except cDNA. Expression of target mRNA was normalized with respect to actin using the ΔΔCT method.

Real-Time RT-PCR was performed by two independent methods for the TC22 transcript (Genebank accession number AY004867) and by one method for the hTM5 transcript (Genebank accession numbers X04588, BC000771, and AK026559). SYBR Green detection was completed for both TC22 and hTM5 transcripts on the *Roche Lightcycler* using the *Qiagen SYBR Green PCR Kit*. All samples underwent 40 cycles of denaturing at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extending at 72°C for 20 seconds. 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'.

Additionally, TaqMan assays were completed for TC22 in parallel with the SYBR assays. TaqMan analysis was performed with the *ABI PRISM 7900 Sequence Detection System* (Applied Biosystems) using *TaqMan Universal PCR Master Mix*. All samples underwent 40 cycles of denaturing at 95°C for 10 seconds, and annealing/extending at

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60°C for 1min. *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 as well as the oligonucleotide probe.

siRNA Against TC22

Silencer select pre-designed siRNA against the TC22 gene product (Genebank

accession number AY004867) were commercially obtained from Ambion (Austin,

Texas). Transfection was performed on $0.1X10^6$ cells per well using siPORT transfection

reagent. Titration experiments identified an optimum concentration of 30nM of siRNA to

effectively silence TC22 protein and gene products. Cells were transfected in parallel

with CY-3 labeled GAPDH to assess transfection efficiency, as well as scrambled siRNA

as a negative control. Medium was changed 24hrs after transfection and cells were

observed for CY3 fluorescence. Cells were harvested at 48hrs and transfection efficiency

was also assayed at this time using GAPDH antibody.

PCR Assay of Carcinogenic Pathways

RT^2 Profiler Human Cancer PathwayFinder 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 as per the

manufacturer's protocol.

Apoptosis

Apoptosis was examined by Vybrant Apoptosis Assay Kit #2 (Molecular Probes,

Netherlands) according to manufacturer's protocol.

Statistical Analysis

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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 standard error of the mean (SEM).

Results

Specificity and Sensitivity of the Monoclonal Antibodies Against hTM5 and TC22

Using recombinant isoforms of hTM (hTM1-hTM5) and TC22, sensitivity and specificity of CG3 and TC22-4 are illustrated by ELISA and Western blot assays (Figure 1A). CG3 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, Figure 1A) and by the visualized bands. As 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 (Figure 1B). All ELISA OD values against hTM 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 (Figure 1A).

Specific Reduction of TC22 Protein by 5-ASA

LS180 colon cancer cells were incubated with 2mM 5-ASA for 0.5 hr, 1 hr, 1.5 hr, 2 hr, 3 hr, 4hr, 8hr, and 24hr. The viability of the cells was above 90% after each incubation. Figure 2A and B show the expression of TC22 and hTM5 at various time

points following incubation with 2mM 5-ASA. There was no significant change in TC22

expression at 0.5 hr or 1 hr (Figures 2A). However, at 1.5 hr, TC22 expression decreased

sharply on average by 45.03% (SEM±1.66, p<0.0117) compared with the 0hr time point

and continued to be decreased by 49.17% (SEM±7.28, p<0.0468) at 2hrs, by 38.03%

(SEM±5.78, p<0.0341) at 3hrs, by 49.10% (SEM±7.03, p<0.0002) at 4hrs, by 45.60%

(SEM±3.87, p<0.027) at 8hrs, and by 49.39% (SEM±5.89, p<0.0002) at 24hrs.

Therefore, a significant 40-50% reduction of TC22 expression was observed starting

within 1.5hrs of treating the cells with 2mM 5-ASA and was sustained for 24hrs without

further addition of the drug (Figure 2A & B). It is important to note that the suppression

of TC22 was specific, as hTM5 expression examined in parallel remained essentially

unchanged (Figure 2A).

This effect on TC22 was found to be dose dependent, as 1mM 5-ASA treatment

reduced TC22, by only 26.91% (SEM±8.79) at 2hrs of treatment. However, 2mM 5ASA

at the same 2hr time point reduced TC22 expression by 49.17% (p<0.0468) (Figure 3A).

We also analyzed whether the effect of 2mM 5ASA on TC22 expression was reversible if

5-ASA exposure is removed, and whether re-treatment with a fresh batch of drug caused

any further effect (Figure 2C displays a representative experiment). When the LS180

cells were incubated with 2mM 5ASA for 4 hrs, washed, replaced with 10mL serum free

DMEM, and harvested after 24hrs, the TC22 suppression was only 11.35% (SEM±2.68,

p<0.0737) compared to pre-treatment value. However if the LS180 cells were incubated

with 2mM 5ASA for 4hrs, washed, and re-treated with fresh 2mM 5ASA solution, the

suppression was 59.02% (SEM±6.336, p<0.0057), greater on average than the

suppression seen with single treatments at 4 hrs (49.10%) or 24hrs (49.39%). Cells were

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also retreated daily with fresh solutions of 2mM 5ASA for up to 5 days, showing a continued suppression of TC22 by 32.96% (SEM±9.07, $p<0.012$) at 72hrs and 46.09% (SEM±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 as well as hTM5 expression *in vitro* (Figure 3B). At 4 hours, we found 2mM SASP significantly reduced TC22 expression by 44.4% (SEM±16.52, $p<0.03$) compared with the pre-treatment value, whereas 2mM SP had no effect. Rosiglitazone (10μM) also significantly reduced TC22 expression by 41.0% (SEM±8.3, $p<0.002$), similar to the level of modulation by 5-ASA and SASP. Neither SASP, SP, nor rosiglitazone affected cellular expression of hTM5 (data not shown). The large SEM of SASP is due to the difficulty in keeping SASP in solution at pH 7.2-7.4. However, the suppression was still significant ($p<0.03$) compared with the pre-treatment value (Figure 3B).

We also treated the cells with 2mM aspirin, because of the structural similarity with 5-ASA. In contrast to 5-ASA, 2mM aspirin decreased TC22 only by 7.31% (SEM±3.8), which was not significantly different than the pre-treatment value. Sulindac (1mM) decreased TC22 by only 13.9% (SEM±5.57, $p<0.070$) (Figure 3B). Thus, neither sulindac nor aspirin significantly affected cellular TC22 or hTM5 expression.

Apoptosis

At 4hrs of treatment with 5ASA or SASP there was no significant change in apoptosis noted (Figure 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 (Figure 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 hrs and 24 hrs of incubation with 5-ASA. These experiments

demonstrated a significant, 50-60% suppression of the TC22 transcript at 4 hrs which was

verified both by SYBR ($p < 0.006$) and Taq Man ($p < 0.018$) assays (Figure 5). At 24 hrs,

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 hrs and

24 hrs (Figure 5).

TC22 Silencing by siRNA and Carcinogenesis PCR Array

LS180 colon cancer cells were transfected with CY-3 labeled GAPDH and were

assessed for efficiency at 48hrs (Figure 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, in comparison to a 2% change in TC22 protein expression in the

scrambled siRNA negative control cells (Figure 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[2] Profiler Human Cancer PathwayFinder PCR Array), in

comparison to untransfected cells, generated the modulations seen in Table 1. Similar

changes were observed when comparing the data from the transfected cells with that from

the scrambled siRNA negative control. Most notably, among the statistically significant

(>2 fold) changes observed, TC22 silencing was predominantly pro-apoptotic with

increases of two TNF superfamily products [TNFRSF10B (+3.1x), TNFRSF25 (+2.0x)] and involved in cell adhesion with increases in two integrin subunits [ITGA1 (+2.6x), ITGA3 (+3.0x)]. In addition, the array indicates that suppression of TC22 may induce angiogenesis [IGF1 (+6.9x), TEK (+12.7x), VEGFA (+3.1x), PDGFA (-2.0x)] as well as two matrix metalloproteinases [MMP1 (+9.2x), and MMP2 (+3.6x)].

Discussion

Our study shows that 5ASA and SASP specifically decrease the cellular expression of TC22, a novel biomarker of colon cancer. The inactive metabolite of SASP, SP, failed to produce any effect on TC22. A concentration of 2mM *in vitro* was chosen because of: measured intraluminal concentrations of 7.0-14.0mM 5-ASA in IBD patients on maintenance therapy (Lauritsen et al., 1984; Allgayer, 2003), titration experiments indicating cell toxicity in culture beyond 10mM, 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 as TC22 is strongly associated with colonic neoplasia and carcinoma but is not detected in normal colon epithelium (Lin et al., 2002). We previously reported that 21/22 colon cancer specimens showed TC22 expression, whereas only 1/17 non-cancer colonic mucosal samples and 0/13 hyperplastic polyps expressed the protein (p<0.0001) (Lin et al., 2002). While the specific physiologic and pathophysiologic role of TC22 remains 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 due to the clinical relevance of the modulation of TNF

molecules in UC as well as the potentially pro-apoptotic 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. While others have shown 5-ASA to induce apoptosis *in vitro* at concentrations in excess of 40mM (Bus et al., 1999), our results suggest that at a clinically relevant dosage of 2mM there was no significant increase in apoptosis. Though 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 while we were able to establish a firm a link between the suppression of TC22 by 5ASA 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 4hrs 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 50-60% ($p < 0.018$ to $p < 0.006$) at 4hrs, however, unlike the protein level, it returns to baseline by 24 hrs. It is possible that though the suppression of the TC22 protein by 5-ASA was sustained for 24 hrs, the effect on the gene is shorter in duration. Accordingly, an increase in protein amount might be observed beyond 24hrs as 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, >1.2g/day,

shown epidemiologically to be particularly chemopreventative (Rubin et al., 2003;

Velayos et al., 2005). The reversibility of TC22 expression following the removal of 5-

ASA *in vitro* and the enhancement of suppression following re-treatment, 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, Rubin et al.,

2003, Velayos et al., 2005; Croog et al., 2003).

SASP contains about one third 5-ASA and two-thirds SP by weight. Therefore at

presumably the "same" experimental concentration of 2mM, SASP significantly (p<0.03)

reduced TC22 by a similar amount as 3 times the dosage, by weight, of 5-ASA (49.10%

for 5-ASA vs. 44.4% for SASP). Thus the suppression of TC22 by SASP seems to be

unique, in that it cannot be explained exclusively due to the effect of the 5-ASA moiety

present in SASP alone. The data raise a 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

due to the SP moiety (Das et al., 1973), and *in vitro* prove to be cytotoxic and difficult to

dissolve at pH 7.4.

It has been suggested that the anti-neoplastic effect of 5-ASA in UC may be due

to its continuous anti-inflammatory effects, which may protect against colorectal cancer.

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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 anti-neoplastic 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 anti-neoplastic 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 (Figure 7). There are also notable differences in terms of availability to the tissue, primarily systemic for aspirin vs. primarily topical for 5-ASA, as well as mode of action, primarily COX mediated for aspirin vs. essentially COX unrelated for 5-ASA. The biologic functions of the two compounds also differ considerably. While 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 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.

Recently, it has been demonstrated that the anti-inflammatory effect of 5ASA may be mediated in part via the PPARγ 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., 2008; Lewis et al., 2001; Girnun and Spiegelman, 2003). Our results indicate that rosiglitazone, a TZD and specific agonist of PPARγ, 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 hours. These data provide evidence that the

anti-neoplastic effect of 5-ASA may be mediated via the induction of the PPARγ

pathway.

Thus, the specific reduction of TC22 expression, a biomarker for colon

carcinogenesis, by SASP, 5-ASA, and the PPARγ agonist rosiglitazone, may provide a

novel molecular mechanism for mesalamine's epidemiologically observed anti-cancer

potential in patients with UC.

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Footnotes:

a) **Grant Support:** This work is supported in part by a research grant RO1

DK63618 from NIDDK (NIH). *KKD received a Student Research Fellowship

award from the CCFA for part of this work.

b) Kiron M. Das, M.D., Ph.D.

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Legends for Figures:

Fig. 1

A. Reactivity of TC22-4 & CG3 Antibodies to hTM Isoforms by ELISA & Western Blot -Western blot analysis of anti-hTM5 (CG3) and anti-TC22 (TC22-4) monoclonal antibodies (mAb) against recombinant hTM isoforms hTM1-5 and TC22 (50 ng/lane). Corresponding ELISA values are shown above each lane. The specificity of the respective mAbs is evident. Since the epitope recognized by CG3 mAb is against aa residues 4-10 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 1-221. However, at the C-terminal domain, aa residues 222-247 are different.

Fig. 2

A. Summary of FACS Data Using 2mM 5-ASA Treatment on TC22 & hTM5 Expression in LS180 Cells - A significant inhibition of TC22 (40-50%) is noted at 1.5hrs that is sustained over 24 hrs. However, hTM5 levels remain essentially unchanged (±10%). Each data point is derived from n=3-10 experiments, mean±SEM.

B. Effect of 2mM 5-ASA on TC22 Expression in LS180 Cells at Various Time Points - A representative example of FACS data of TC22 expression following exposure to 5-ASA (2mM). 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.

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C. Reversibility & the Effect of Retreatment with 2mM 5-ASA on TC22 Expression in LS180 Cells - The suppression of TC22 is reversible following removal of the drug at 4 hrs and incubation with serum free medium for 24 hrs ("Reversibility"). However, retreatment of the cells at 4hrs with a fresh solution of 5-ASA (2mM) showed further suppression of TC22 expression ("Retreatment").

Fig. 3

A. Dose Response of 5-ASA on TC22 - Dose response of 1mM and 2mM 5-ASA on TC22 expression at 2hrs. 2mM 5ASA 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 hrs - TC22 expression in LS180 cells at 4hrs of treatment with Aspirin (2mM), Sulfapyradine (SP) (2mM), Sulindac (1mM), Rosiglitazone (Rosi) (10µM), Sulfasalazine (SASP) (2mM), and 5-ASA (2mM) by FACS. Significant suppression was evident with 5-ASA, SASP, and Rosiglitazone but was not found with Aspirin, SP, and Sulindac (n=3 experiments).

Fig. 4 – **Early and Late Apoptosis Upon Exposure of LS180 Cells to 2mM 5ASA or 2mM SASP for 4hrs -** Left hand panel shows the mean value (±SEM) of early apoptosis and late apoptosis from 3 experiments. The right hand panel shows the FACS profile demonstrating a rightward shift toward greater PI and Annexin V positivity. No statistically significant differences in apoptosis were noted when compared to untreated cells.

Fig. 5 – **Real Time RT-PCR for hTM5 & TC22 Transcripts in LS180 Cells Treated with 2mM 5-ASA** – A significant reduction of the TC22 transcript at 4 hours was measured by both SYBR ($p<0.006$) and TaqMan ($p<0.018$) assays (n=3). The level of

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TC22 transcript returned to baseline at 24 hours. Note that the hTM5 transcript did not

significantly change at 4 or 24 hours.

Fig. 6 – **Silencing of TC22 by siRNA**

A. Efficiency of siRNA Transfection - Cy3 -TC22 siRNA transfected LS180 cells seen

by phase contrast and fluorescence (45x), 48 hours 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 in comparison to the untransfected

control. 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, scaled, after

subtraction of MOPC IgG profile by the Overton method.

Fig. 7 – Structure of Various Compounds Employed

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Table 1 – **Effect of TC22-siRNA on Gene Level Changes Related to Carcinogenesis**

	Gene	Description	Fold
Apoptosis Signaling	TNFRSF10B	TNF-receptor superfamily containing intracelluar death domain that transduces apoptosis.	+3.1x
	TNFRSF25	TNF-receptor superfamily, possibly involved in regulating lymphocyte homeostasis. Stimulates NF-kappa B activity and regulates cell apoptosis.	+2.0x
Cell Adhesion	ITGA1	Integrin, Alpha 1	+2.6x
	ITGA3	Integrin, Alpha 3 (CD49C)	+3.0x
Angiogenesis	IGF1	Insulin-like Growth Factor 1 / Somatomedin C	+6.9x
	TEK	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.	+12.7x
	VEGFA	Vascular endothelial growth factor A	+3.1x
	PDGFA	Platelet derived growth factor A	-2.0x
Matrix Metalloproteinases	MMP1	Breakdown of extracellular matrix in embryonic development and reproductive tissue remodeling.	+9.2x
	MMP2		+3.6x

Figure 1

A.

	TC22	hTM1	hTM2	hTM3	hTM4	hTM5
CG3	0.156	0.002	0.004	0.006	0.015	**2.038**
TC22-4	**>4.000**	0.003	0.002	0.003	0.003	0.005

B.
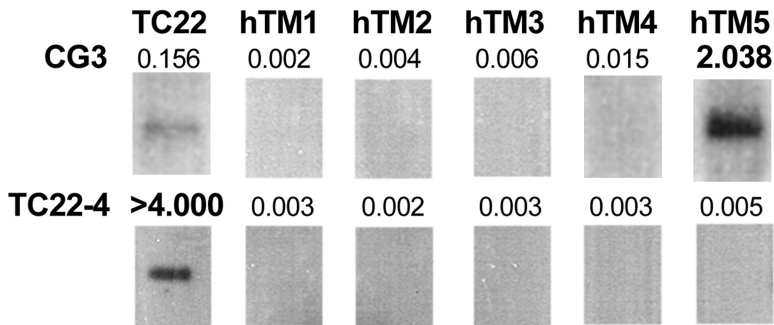
hTM5 222   248

hTM5 K L K C T K E E H L C T Q R M L D Q T L L D L N E M

TC22 222   247

TC22 R L Y S Q L E R N R L L S N E L K L T L H D L C D

Figure 2

A

B

C

Figure 3

A

0 hr   2 hr

Percent Change

1mM 5ASA

2mM 5ASA
*p<0.0468

B

Percent Change

2mM ASA

2mM SP

1mM Sulindac

10uM Rosi
*p<0.002
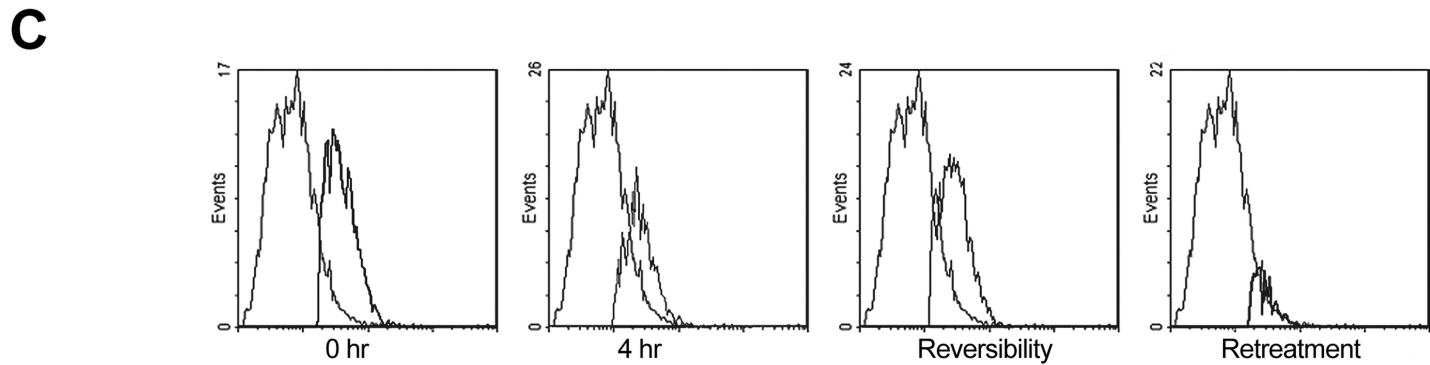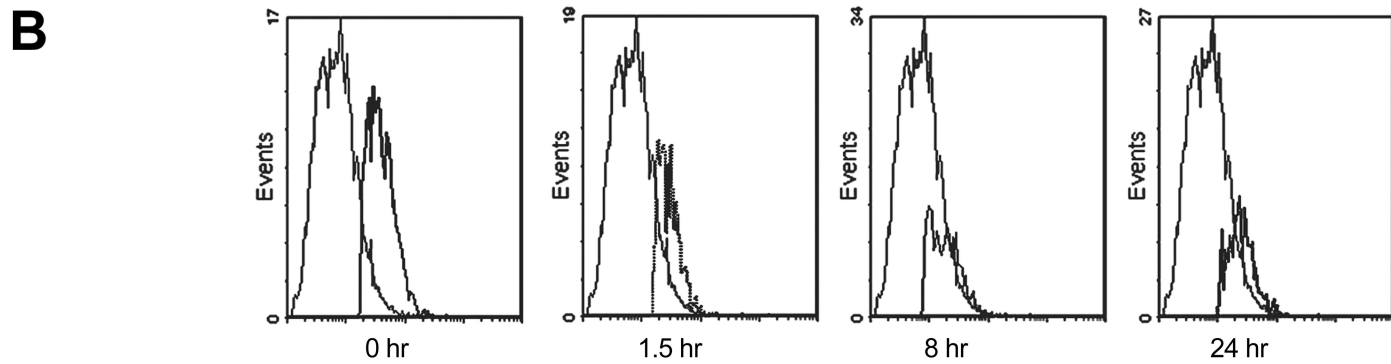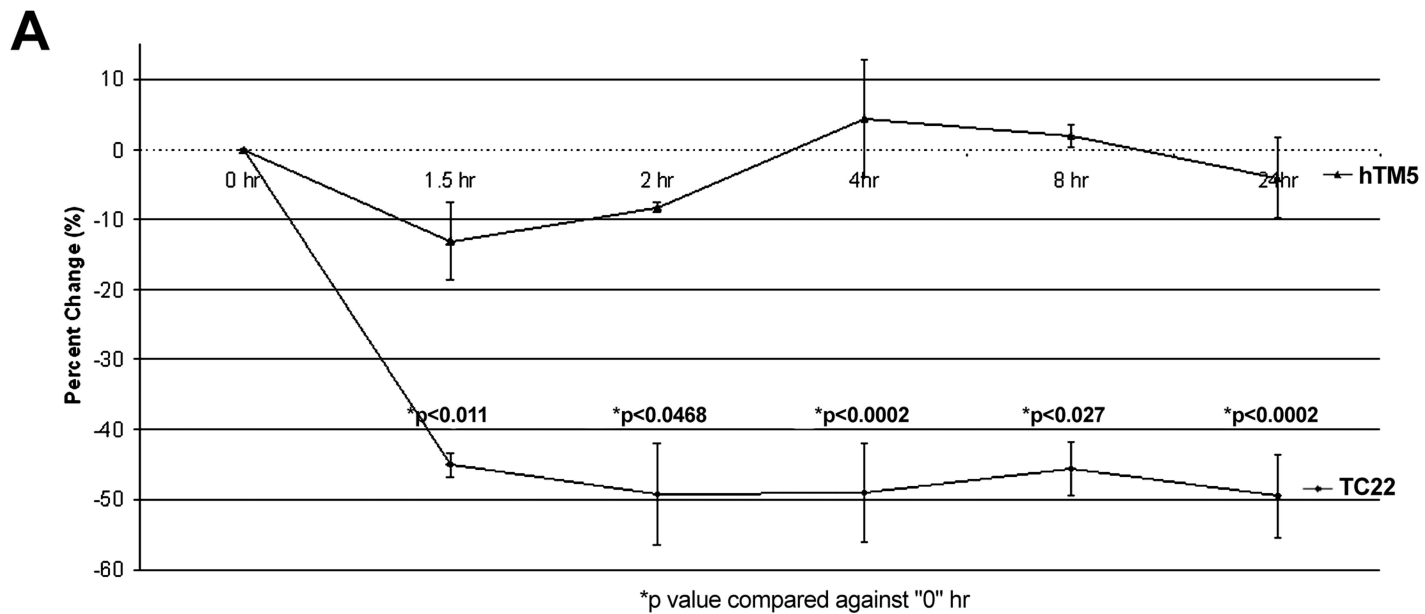
2mM SASP
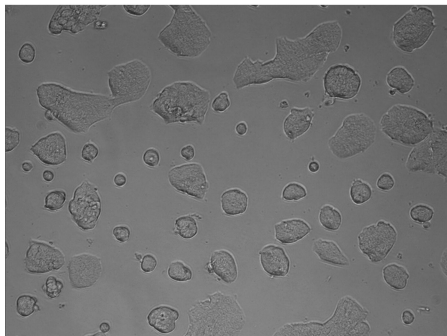*p<0.03
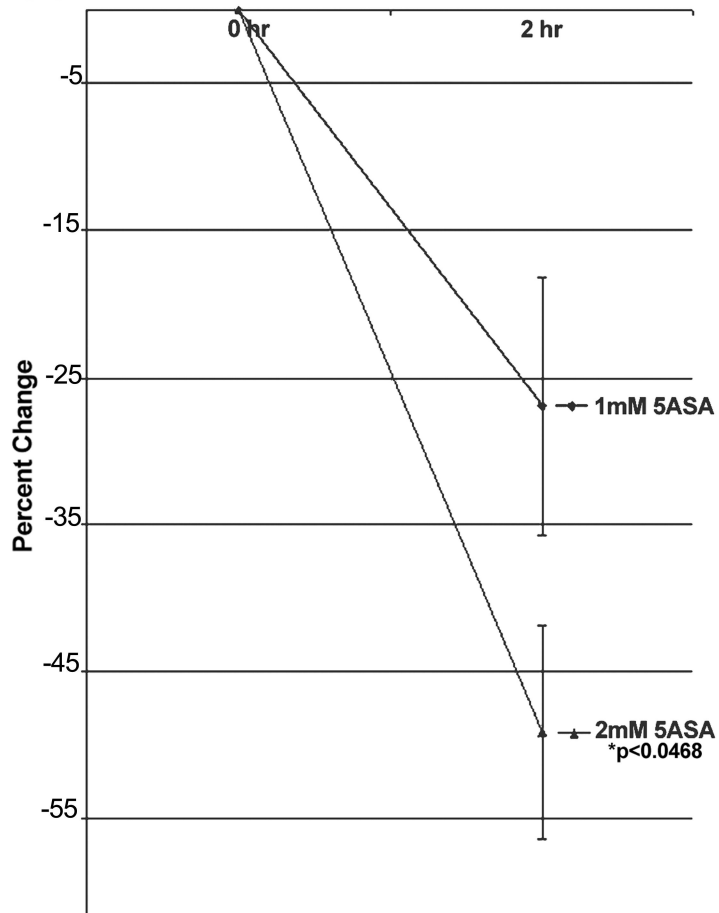
2mM 5ASA
*p<0.002

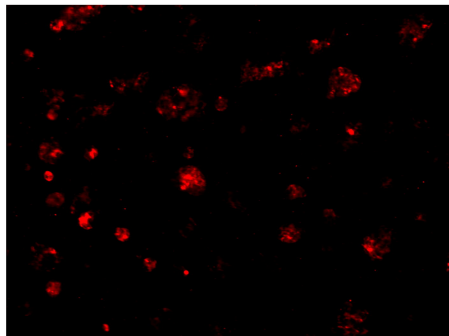*p values compared against "0" hr
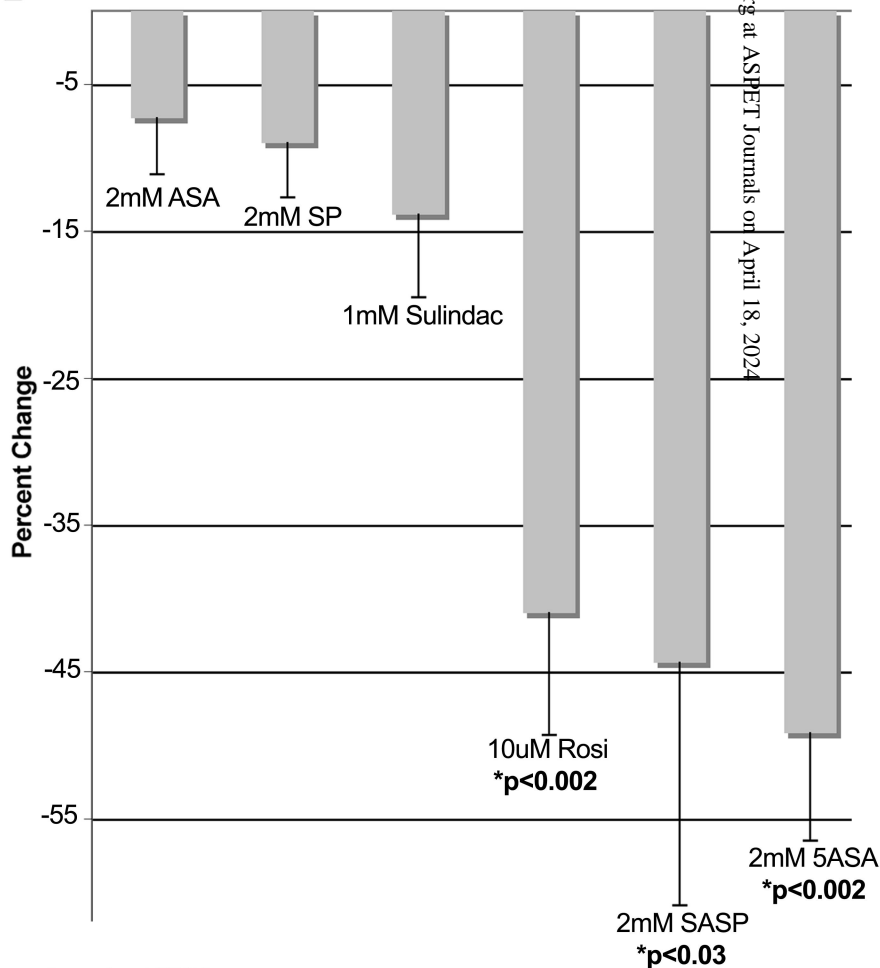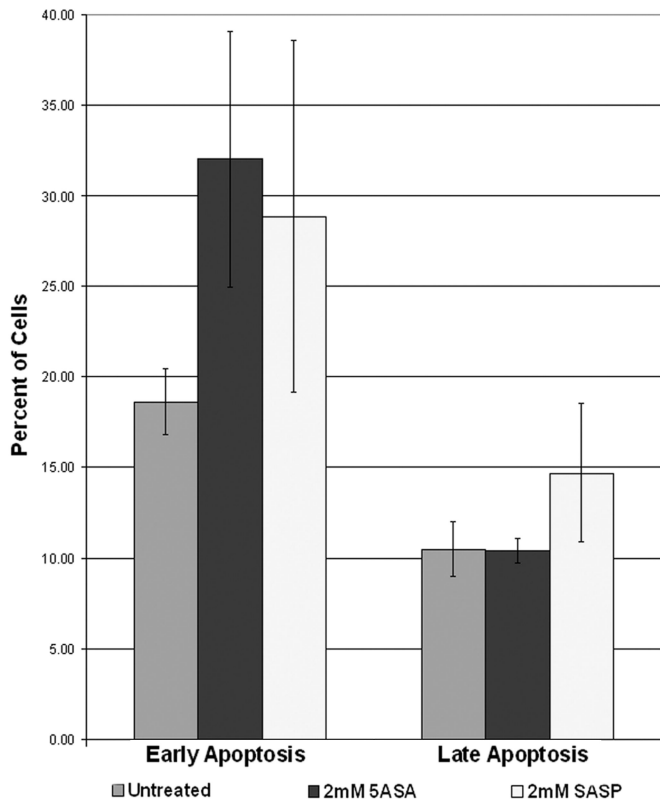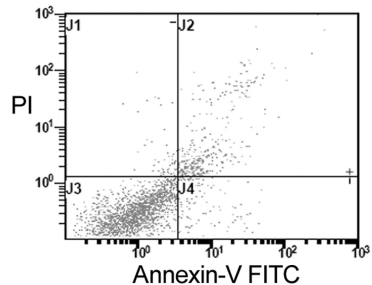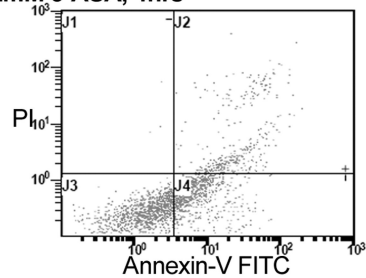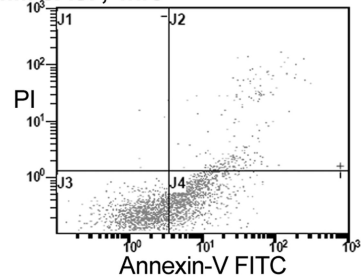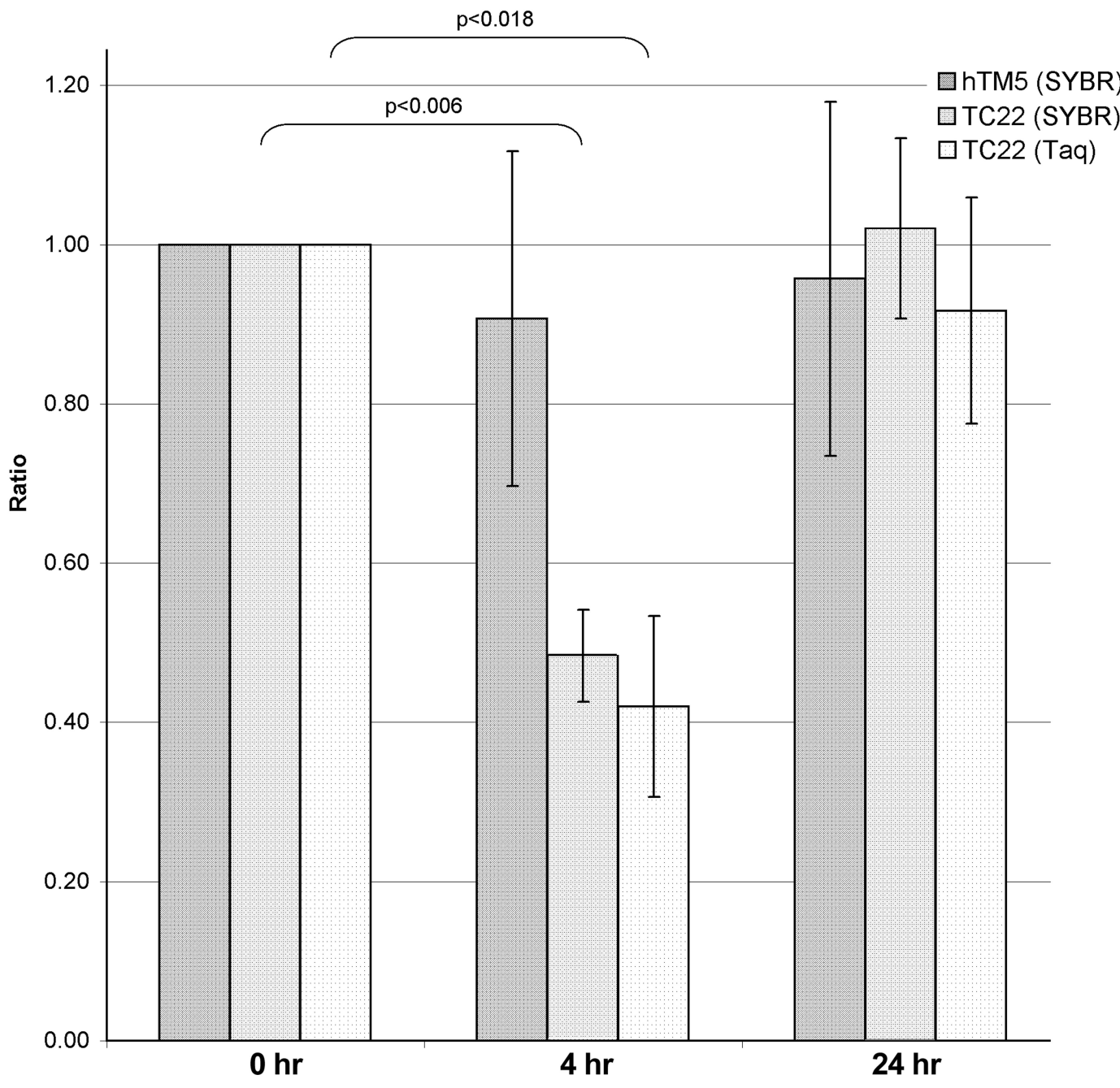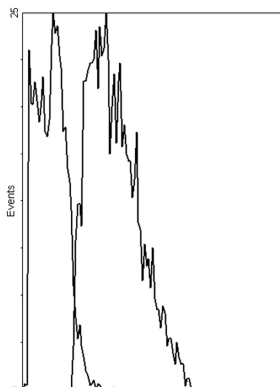
Figure 4

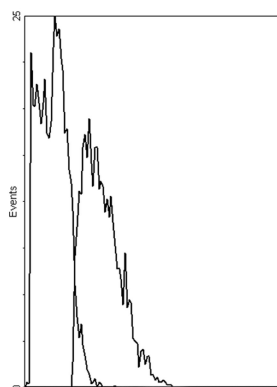# Figure 5

Figure 6

A

Phase Contrast (40x)

Fluoresence (40x)

B

Untreated LS180

TC22-siRNA Transfected LS180

Figure 7

Sulfasalazine

Sulfapyridine

5-Aminosalicylic Acid
(Mesalamine)

Sulindac

Aspirin

Rosiglitazone