Chapter 6

5-Aminosalicylic acid inhibits TGF-β1 signalling in colorectal cancer cells

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Abstract

The Transforming Growth Factor-β (TGF-β) pathway is an important pathway in the initiation and progression of colorectal cancer. We aimed to determine the effects of 5-Aminosalicylic acid (5-ASA) on TGF-β signalling in colorectal cancer cells *in vitro*. 5-ASA inhibited TGF-β1 signalling in HCT116 cells and colonic fibroblasts, as judged by a TGF-β-specific reporter gene assay, plasminogen activator inhibitor-1 mRNA and protein levels, fibroblast trans-differentiation, Smad3 phosphorylation and nuclear translocation. We conclude that 5-ASA inhibits TGF-β1 signalling in colorectal cancer cells, and might be a potent adjuvant therapeutic drug, interfering with aberrant TGF-β signalling in colorectal cancer.
Introduction

The transforming growth factor-β (TGF-β) superfamily consists of TGF-βs, bone morphogenetic proteins (BMPs) and activins. These polypeptides play pivotal roles in intercellular communication, cell proliferation, cell motility, functional differentiation and apoptosis. TGF-β is an important player in malignant diseases, including colorectal cancer (CRC). Most CRCs are resistant to TGF-β-induced growth inhibition and usually show a high TGF-β expression. TGF-β acts as a tumour promoter during the later stages of colorectal carcinogenesis, via promotion of tumour angiogenesis, increased production of extracellular matrix (ECM) and proteolytic enzymes, increased cancer cell motility and immunosuppression. Moreover, TGF-β drives the transdifferentiation of fibroblasts into myofibroblasts, which are abundantly present in colorectal carcinomas and interact with tumour cells to contribute to cancer progression and metastasis.

TGF-β binds to a heteromeric complex of transmembrane kinase receptors, TGF-βR-I and TGF-βR-II. Upon TGF-β binding to TGF-βR-II, TGF-βR-I is recruited to the receptor complex. This allows the TGF-βR-II to transphosphorylate the TGF-βR-I, activin-receptor-like-kinase-5 (ALK-5), which in turn phosphorylates the Smad regulatory proteins, Smad2 and Smad3. These phosphorylated Smads associate with the co-Smad, Smad4, and the complex translocates to the nucleus, interacting with other transcription factors, in a cell specific manner, to regulate a panel of TGF-β-responsive genes, like plasminogen activator inhibitor-1 (PAI-1).

Blockage of aberrant TGF-β signalling in CRC interrupts multiple events that are important for tumour maintenance and progression, and PAI-1 blockers have been shown to reduce polyp formation in mice. Several non-steroid-anti-inflammatory drugs (NSAIDs) have been reported to possess anti-colorectal cancer capacities. 5-Aminosalicylic acid (5-ASA) is such an NSAID, used in the treatment of inflammatory bowel diseases (IBD), that has anti-CRC properties, although the mode of action is only partially understood.

In the present study we assessed whether 5-ASA affects the TGF-β response of colorectal cancer-derived cells and illustrate mechanisms involved. 5-ASA was found to reduce TGF-β1 signalling in the CRC cells as judged by the reduction in TGF-β specific reporter gene activity, PAI-1 mRNA and protein levels, fibroblast differentiation, Smad3 phosphorylation and nuclear translocation.
Material&Methods

Cell culture and reagents

HCT116, HT29, LS180 and HepG2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM)/F12 (1:1) + GlutaMAX supplemented with 10 mM HEPES, 100 U/ml penicilin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (all Invitrogen, Breda, The Netherlands) and 10 % heat inactivated Fetal Calf Serum (FCS, Perbio Science, Erembodegem, Belgium). Stock solutions of 5-ASA (Dr. Falk Pharma, Freiburg, Germany) were prepared fresh in culture medium. 5-ASA was dissolved in culture medium, adjusted to pH 7.4 and filter-sterilized. Salofalk enemas (4 gram 5-ASA/60 gram, Tramedico BV, Weesp, The Netherlands) were emptied and stock solutions were made similar as described above. GW9662 (Calbiochem, San Diego, CA, USA), an antagonist of the peroxisome proliferator-activated receptor-γ (PPARγ), was dissolved in dimethyl sulfoxide (DMSO) in a 10 mM stock solution. The ALK-5 inhibitor SB-421543 (Sigma-Aldrich, Germany) was used at a 10 µM concentration.

Fibroblasts were isolated from human colon carcinoma and normal tissue as described before 21, and maintained in complete DMEM/F12 containing 10% FCS and used at passage 5-11.

TGF-β and BMP response assays

The Smad3/4 specific (CAGA)_{12}-MLP-Luciferase reporter plasmid consists of 12 tandem repeats of the upstream Smad3/4 binding element of the human PAI-1 promoter linked to luciferase 22. To measure TGF-β signalling we plated HCT116 cells in 24 well plates (Corning) and transiently transfected the cells with (CAGA)_{12}-MLP-Luc reporter plasmids (0.4 µg) and β-galactosidase plasmid (0.1 µg) using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s protocol. Transfected cells were serum starved for 16-18 hours and treated with 5 ng/ml recombinant human TGF-β1 (Reprotech, London, UK) in serum free culture medium for 6 hours, or different conditions as indicated. After stimulation the cells were washed, lysed and luciferase activity was determined according to the manufacturer’s protocol (Promega, Madison, WI, USA). β-galactosidase activity in the lysates was determined using β- galactosidase substrate (0.2M H₂PO₄, 2 mM MgCl₂, 4 mM ONPG, 0.25% β-mercaptoethanol) and spectrophotometrically read at 405 nm. The luciferase count was corrected for β-galactosidase activity and the relative increase in corrected luciferase count was calculated versus controls. A constitutively active ALK5 (caALK5)
construct was co-transfected in a concentration of 0.2 μg per well, in the indicated experiments.

To measure BMP signalling cells were transfected with a BMP specific response plasmid (BRE-Luc) \(^{23}\), serum starved and stimulated with 50 ng/ml recombinant human BMP-2 (R&D systems, Oxford, UK), similar to the TGF-β protocol above.

Fibroblasts were infected using an adenoviral Ad-(CAGA)\(^{9}\)-MLP-Luc promoter reporter construct \(^{24}\). Cells (18,000/well) were infected with 1x10\(^6\) pfu virus. After infection, medium was changed to complete DMEM/F12 for 24 hours and the fibroblasts were serum starved overnight, stimulated, lysed and the luciferase activity was determined as described above.

**RNA isolation and Real Time PCR**

HCT116 cells (5 x 10\(^5\)) were seeded into 6-well plates and allowed to adhere for 24 hours. Cells stimulated with 5 ng/ml TGF-β1 for 6 hours and collected by trypsinisation. RNA was isolated according to miniprep protocol (Qiagen, Germantown, MD, USA). RNA concentration and purity was checked by A260/A280 optical density ratio. cDNA was generated from 1 μg RNA, using random primers, and subjected to 40 cycles Real-time PCR (RT-PCR) analysis. mRNA levels were calculated relative to glyceraldehyde phosphate dehydrogenase (GAPDH), which was unaffected by 5-ASA and TGF-β1 treatment. Probes and primers were obtained from Isogen (Maarsen, The Netherlands) and Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands).

**Nuc ear and cytoplasmic extract isolation**

HCT116 cells were grown in 75 cm\(^2\) flasks, serum starved for 16-18 hours and incubated in serum-free DMEM/F12 containing TGF-β1 (5 ng/ml), 5-ASA (30 mM) and/or SB-421543 (10 μM) for 60 minutes. Nuclear and cytoplasmic protein fractions were separated by a commercially available separation kit (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Protein concentrations were determined using the BCA assay (Pierce).

**Western blot analysis**

HCT116 cells were grown in 24 well plates, serum starved for 16-18 hours, and subsequently stimulated with TGF-β1 (5 ng/ml), 5-ASA (30 mM) and/or SB-421543 (10 μM) during a time course. The lysates were subjected to 10% SDS-PAGE and blotted to
nitrocellulose membranes (Whatman, Dassel, Germany). Blots were blocked with 2.5% non-fat dry milkpowder (Bio-Rad laboratories, Hercules, CA, USA) in PBS containing 0.05% Tween 20 (PBST) for 2 hours at room temperature (RT) and afterwards incubated overnight at 4°C with primary antibodies in 0.5 % BSA (Sigma-Aldrich) in PBST [Smad2 (1:1000, Santa cruz, S-20), Smad3 (1:1000, Zymed Laboratories, USA), p-Smad3 (1:5000, provided by Ed Leof (Mayo Clinic, Rochester, Minnesota, USA), Poly (ADP-ribose) polymerase (PARP, 1:5000, Calbiochem), β-actin (1:10.000, Sigma-Aldrich), GAPDH (1:1000, Millipore, USA), α-smooth muscle actin (α-SMA, 1:1000, Progen, Heidelberg, Germany)]. After subsequent incubation with horse-radish peroxidase(HRP)-conjugated secondary antibodies (Dako, Glostrup, Denmark, 1:1000) for 2 hours in 0.5 % BSA in PBST, the antibodies were visualized with Supersignal West Dura Extented Duration Substrate (Pierce) on a Bio-Rad system. Blots were afterwards stripped with restore stripping buffer (Pierce) and reprobed with antibodies to check equal loading.

Immunofluorescent detection of Smad2/3 and αSMA

HCT116 cells were grown on collagen I (PureCol™, Inamed Biomaterials, Fremont, USA) coated coverslips. After 16-18 hours of serum starvation, cells were stimulated with TGF-β1 (5 ng/ml) with or without 30 mM 5-ASA for 60 minutes. Cells were washed with phosphate buffered saline (PBS) and fixed in 4 % buffered formaldehyde for 10 minutes. After washings with PBS, cells were permeabilized with 0.5 % TritonX-100 in PBS and blocked with 5% goat serum (Dako) in 1 % BSA/PBS. After incubation with mouse-anti-Smad2/3 (1:200, BD Transduction Laboratories, USA) in 1% BSA/PBS for 2 hours at RT, cells were incubated with goat-anti-mouse-Alexa594 (Invitrogen) at RT for 1 hour. Coverslips were mounted with slow fade gold antifade reagent with DAPI (Invitrogen).

Fibroblasts were also grown on coverslips and stimulated with 5 ng/ml TGF-β1 in 10% FCS medium for 24 hours. Cells were washed with PBS and fixated in icecold acetone for 10 min. After washings with PBS and blocking with 5% goat serum in 1% BSA/PBS cells were incubated with mouse-anti-α-SMA (1:1000, Progen, Heidelberg, Germany) and goat-anti-mouse-FITC (Sigma-Aldrich), similar to the protocol above.

Photomicrographs were taken with a Nikon Eclipse E800 microscope equipped with a Nikon DXM 200 camera.
PAI-1 ELISA

PAI-1 was detected in HCT116 cell lysates by a sandwich ELISA that detects latent (inactive) PAI-1, active PAI-1, and PAI-1 complexes (American Diagnostica Inc, Stanford, CT, USA).

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). Student’s t-test were used to compare TGF-β1 conditions with or without 5-ASA and differences with P ≤ 0.05 were considered significant.

Results

5-ASA blocks TGF-β1-induced transcriptional responses

HCT116 cells were selected out of panel of CRC cell lines to examine the effect of 5-ASA on TGF-β1 signalling. They show a good dosage-dependent responsiveness to TGF-β1, as determined by transient transfection assays using the TGF-β-responsive luciferase promoter construct (CAGA)$_{12}$-Luc, as observed in about 50% of the CRC cell lines (Hawinkels et al, submitted). TGF-β1 (5 ng/ml) induced (CAGA)$_{12}$-Luc activity about 6 fold in HCT116 cells (Figure 1A). The TGF-β1-dependent luciferase activity was reduced by simultaneous 5-ASA treatment in a concentration dependent manner, with a ~ 85 % reduction in 30 mM 5-ASA treated cells (Figure 1A).

Inhibition of the TGF-β1 response by 5-ASA was also observed in other TGF-β1-responsive CRC cell-lines (HT29 and LS180, Figure 1B). 5-ASA did not affect the basic reporter gene activity, indicating that the effect was on the induced TGF-β1 response and not on luciferase activity or cellular activity itself. There was no induction of cell death due to the 6 hour treatment with 30 mM 5-ASA as determined by caspase-3 activity measurement and trypan blue cell exclusion assays (not shown). 5-ASA enemas that are clinically applied were able the block TGF-β1-induced (CAGA)$_{12}$-Luc activity in a similar dose-dependent manner (not shown).
Figure 1: 5-ASA reduces the transcriptional response to TGF-β1.

HCT116 cells transiently effected with the (CAGA)$_{12}$-MLP-Luciferase reporter plasmid show a 6-fold response to 5 ng/ml TGF-β1 which is dose-dependently reduced by 5-ASA treatment (A). HT29 and LS180 also show a response to 5 ng/ml TGF-β1 which is also reduced by 5-ASA (30 mM) or SB-431542 (10 μM) treatment. (B). Colonic fibroblasts show a 9-fold response to 5 ng/ml TGF-β1, which is also dose-dependently reduced by 5-ASA treatment (C). * P<0.05, ** P<0.01.

To determine the effects of 5-ASA on TGF-β1 signalling in other cell types we used fibroblasts isolated from normal and CRC tissue, that show about a 9-fold up-regulation of (CAGA)$_9$-Luc upon stimulation with TGF-β1 (Figure 1C). 5-ASA treatment also reduced the TGF-β1 response in these fibroblasts in a dose-dependent manner (Figure 1C).

Given the fact that 5-ASA is a ligand for PPARγ, that is able to activate downstream signalling 25, and PPARγ-agonists have been reported to repress TGF-β signalling in other cell types 26, 27, we investigated whether 5-ASA repressed TGF-β signalling in a PPARγ dependent manner. To that end, we treated HCT116 cells with the PPARγ antagonist GW9662, which was unable to rescue the 5-ASA repressing effects on TGF-β1 signalling in
HCT116 cells (Figure 2A), indicating that the 5-ASA effect on TGF-β1 signalling is PPARγ independent.

The specificity of the effect of 5-ASA on TGF-β1 signalling was investigated by comparison with BMP signalling, a closely related pathway. As the majority of CRC cells, including HCT116, have defects in the BMP signalling pathway 28 we used HepG2 cells. TGF-β1 (5 ng/ml) showed a massive activation of (CAGA)12-Luc (~300 fold) in HepG2 cells and 5-ASA also repressed this response by 60 % in these cells (Figure 2B).

**Figure 2:** The 5-ASA effects are PPARγ independent and specific for TGF-β1.

The PPARγ-antagonist GW9662 (μM) is unable to rescue the 5-ASA repressive effect on the TGF-β1 response in HCT116 cells (A). 5-ASA is able to reduce the massive TGF-β1 response in HepG2 cells (B), while leaving the BMP response to BMP-2 (50 ng/ml) unaffected, as indicated by transient transfection assays using BRE-Luc (C). Data represent 3-4 independent experiments, mean ± SEM. Significant differences were calculated with Student’s t-tests, * P<0.05, ** P<0.01, *** P<0.001.

BMP-2 (50 ng/ml) showed a 25-fold induction of BRE-luc, a BMP-responsive luciferase promoter construct, which was hardly affected (only 20 %) by 5-ASA treatment (Figure
These data indicate that 5-ASA reduces TGF-β1 signalling but does not affect the closely related BMP signalling.

5-ASA blocks cellular responses to TGF-β

To examine the downstream effect of 5-ASA on TGF-β signalling we determined the expression of the most prominent TGF-β target gene, PAI-1, in HCT116 cells by RT-PCR. TGF-β1 induced expression of PAI-1 mRNA about 7-fold in HCT116 cells, which was inhibited by 5-ASA in a dose dependent manner, confirming the experiments with (CAGA)$_{12}$-Luc (Figure 3A). The inhibiting role of 5-ASA on PAI-1 expression was confirmed on the protein levels (Figure 3B).

TGF-β1 induced differentiation of fibroblasts into myofibroblasts, as identified by the expression of α-smooth muscle actin (α-SMA), the myofibroblast marker (Figure 2C/D). This trans-differentiation was also inhibited by 5-ASA treatment, indicated by a decrease in the TGF-β1-induced α-SMA expression, demonstrated on western blot and by immunofluorescence (Figure 3C/D).

These results indicate that 5-ASA is able to repress/block TGF-β1-induced responses in CRC cells and fibroblasts.
5-ASA inhibits TGF-β1 signalling in CRC cells in vitro

A

HCT116

B

HCT116

C

Normal colonic fibroblasts

D

Normal colonic fibroblasts

Control  TGF-β1  TGF-β1 + 5-ASA

α-SMA

Hoechst

Merged

α-SMA

Hoechst

Merged
5-ASA suppresses TGF-β1-induced Smad nuclear translocation by reducing Smad3 phosphorylation

Because 5-ASA oxidizes in culture medium, which can have consequences for the activity of peptides, we excluded a direct effect of 5-ASA on the activity of the exogenously added TGF-β1. We therefore pretreated HCT116 cells with 5-ASA for 6 hours and stimulated with 5 ng/ml TGF-β1 afterwards. Pretreatment with 30 mM 5-ASA reduced the subsequent TGF-β1-induced (CAGA)12-Luc activity in HCT116 cells, indicating that the reduction is not due to effects on the activity of TGF-β1, as TGF-β1 and 5-ASA were not simultaneously present in the assay (Figure 4A).

![Graph A](image)

Figure 4: 5-ASA reduces the TGF-β1 response downstream of ALK5.

5-ASA pre-treatment reduces the response to 5 ng/ml TGF-β1 in HCT116 cells (A). HCT116 cells transfected with caALK5 show a massive response in the absence of exogenously added TGF-β. 5-ASA is able to reduce this response similar to the ALK5 inhibitor, SB-421543. Data represent 4-5 independent experiments, mean ± SEM. Significant differences were calculated with Student’s t-tests, * P<0.05 (B). TGF-β1 induced phosphorylation of Smad3 in time, which is 5-ASA reduced by 5-ASA, similar to SB-421543, as determined by western blot (C).

To further analyze if 5-ASA acts on ligand binding and subsequent active receptor complex formation or on downstream TGF-β signalling HCT116 cells were transiently transfected with constitutively active ALK5 (caALK5). This resulted in a ~80-fold induction of (CAGA)12-Luc activity without TGF-β stimulation (Figure 4B). 5-ASA inhibited this to a
5-ASA inhibits TGF-β1 signalling in CRC cells in vitro

5-ASA inhibits TGF-β1 signalling in CRC cells in vitro. Thus, 5-ASA affects components of the TGF-β1-Smad signalling pathway downstream of the receptor complex.

Smad2 and Smad3 are the intracellular mediators of TGF-β signalling that are activated by phosphorylation through ALK5 upon TGF-β stimulation. TGF-β1 treatment increased phosphorylation of Smad3 in HCT116 cells (Figure 4C), which was reduced by 5-ASA, indicating that 5-ASA inhibited ALK5 (Figure 4C). SB-431542 shows similar inhibition of TGF-β-induced Smad3 phosphorylation (Figure 4C).

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Figure 5: 5-ASA blocks TGF-β induced Smad nuclear localization.

TGF-β1 induces a translocation of Smad2/3 towards the nucleus after 60 minutes, which is blocked by 5-ASA, as judged by immunofluorescent detection of Smad2/3 (A, original magnification 1000 x, scale bars: 5 μm, full-colour image on page 185) and the detection of Smad 2 and Smad 3 on separated nuclear and cytoplasmic cellular fractions by western blot (B). PARP and GAPDH were used as loading controls for nuclear and cytoplasmic fractions respectively.
Next we investigated the translocation of phosphorylated Smads, as they translocate to the nucleus to activate transcription. Immunofluorescent detection indicated that the TGF-β1 stimulation induced nuclear translocation of Smad2 and Smad3 after 60 minutes in HCT116 cells, which was prevented by simultaneous treatment with 5-ASA (Figure 5A). Western blotting of separated nuclear and cytosolic protein fractions confirmed these results (Figure 5B).

**Discussion**

In the present study we found that 5-ASA efficiently and specifically blocked the response of CRC cells to TGF-β1 in vitro, in a dose dependent manner, without influencing the closely related BMP signalling pathway. There are several indications in the literature that in normal tissue and pre-malignant cancer stages TGF-β plays a tumour suppressive role, by inhibiting growth and inducing apoptosis, whereas in later stages tumour cells become resistant to these growth inhibitory properties and TGF-β can promote cancer-progression by stimulating migration, invasion and angiogenesis. TGF-β stimulates the expression of several matrix remodeling proteases and inhibitors like PAI-1, which contributes to the tumour progressive role of TGF-β. TGF-β levels and activity are enhanced in CRC and correlate with tumour progression and worse disease prognosis. The HCT116 colonic cancer cells that were mainly used in our experiments were confirmed to be insensitive to the growth reducing capacity of TGF-β (not shown) and indeed showed an increase of PAI-1 expression upon TGF-β stimulation. We therefore investigated 5-ASA’s properties to interfere in TGF-β signalling in these HCT116 CRC cells.

The involvement of TGF-β in tumour progression makes clinically useful antagonists highly desirable. Inhibition of TGF-β signalling by anti-TGF-β-antibodies, soluble TGF-β receptors, anti-sense RNA and TGF-β-RI inhibitors indeed reduce tumour progression, but in view of the important role of TGF-β in other processes, e.g., bone formation and wound healing, these therapies might be accompanied by unwanted side-effects. 5-ASA medication is already used in the treatment of IBD patients since the 1950s, with serious side-effects seldom occurring. Because IBD patients using 5-ASA medication have a decreased risk of developing CRC, 5-ASA has received interest from CRC researchers. Several recent publications indicate that 5-ASA possesses anti-CRC effects, although the working mechanism of the drug has not yet been fully elucidated. We therefore investigated 5-ASAs properties to interfere with TGF-β signalling in CRC cells. We showed that 5-ASA reduced TGF-β1 signalling in HCT116 cells, as judged by a reduction in TGF-β specific
5-ASA inhibits TGF-β1 signalling in CRC cells in vitro

reporter gene activity, PAI-1 mRNA and protein levels. We have recently shown that active TGF-β1 levels are indicative for malignant transformation of CRC and that these levels correlate with myofibroblasts content in CRC. In addition, we observed that 5-ASA was able to efficiently inhibit the TGF-β1 response in CRC-associated fibroblasts and the TGF-β-induced trans-differentiation of fibroblasts into myofibroblasts. TGF-β1 is able to elevate its own expression and activity in cancer cells by interactions with myofibroblasts in a cancer-progressing feedback loop (Hawinkels et al., submitted). Our results indicate that 5-ASA treatment could reduce TGF-β1 levels due to blockade of the feedback loop. 5-ASA medication has indeed been reported to reduce TGF-β1 levels in the circulation and inflamed colorectal tissue of IBD patients.

5-ASA was able to reduce TGF-β signalling downstream of ALK5 in HCT116 cells indicated by the ability to reduce signalling from caALK5, reduction of Smad3 phosphorylation, and reduction of the TGF-β-induced nuclear translocation of Smad2 and 3. High concentrations of 5-ASA (0-50 mM) can be achieved locally in the colorectal lumen upon 5-ASA treatment either by enema and/or coated tablets, while systemic levels remain relatively low. Because 5-ASA is only able to block TGF-β-induced signalling at high concentrations (>10 mM) it is likely that 5-ASA would only inhibit the enhanced TGF-β activity in the colorectal carcinoma, without causing side-effects due to inhibition of normal TGF-β signalling at other sites. Future studies to understand the effect of 5-ASA therapy on TGF-β signalling in the various stages of CRC progression and metastasis may lead to a better understanding of TGF-β signalling in CRC progression and, in combination with current therapies, to a decreased incidence of cancer metastasis.

In conclusion, in the present study we have shown that 5-ASA is able to block the TGF-β response of CRC cells, including cancer-associated fibroblasts, in vitro. This anticancer effect of 5-ASA, together with compelling evidence of anti-cancer effects of 5-ASA described in other studies, renders 5-ASA to be a potentially powerful drug to use as adjuvant therapy to treat CRC.

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References


Chapter 6