Published in final edited form as:

Gastroenterology. 2007 January ; 132(1): 221–235.

5-ASA Affects Cell Cycle Progression in Colorectal Cells by Reversibly Activating a Replication Checkpoint

M. GLORIA LUCIANI^{*}, CHRISTOPH CAMPREGHER^{*}, JOHN M. FORTUNE[‡], THOMAS A. KUNKEL[‡], and CHRISTOPH GASCHE^{*}

^{*} Medical University of Vienna, Department of Internal Medicine IV, Division of Gastroenterology and Hepatology, Vienna, Austria

[‡] Laboratory of Molecular Genetics and Laboratory of Structural Biology, National Institute of Environmental Health Sciences, Research, Triangle Park, North Carolina

Abstract

Background & Aims— Individuals with inflammatory bowel disease are at risk of developing colorectal cancer (CRC). Epidemiologic, animal, and laboratory studies suggest that 5-amino-salicylic acid (5-ASA) protects from the development of CRC by altering cell cycle progression and by inducing apoptosis. Our previous results indicate that 5-ASA improves replication fidelity in colorectal cells, an effect that is active in reducing mutations. In this study, we hypothesized that 5-ASA restrains cell cycle progression by activating checkpoint pathways in colorectal cell lines, which would prevent tumor development and improve genomic stability.

Methods— CRC cells with different genetic backgrounds such as HT29, HCT116, HCT116^{p53^{-/-}}, HCT116+chr3, and LoVo were treated with 5-ASA for 2–96 hours. Cell cycle progression, phosphorylation, and DNA binding of cell cycle checkpoint proteins were analyzed.

Results— We found that 5-ASA at concentrations between 10 and 40 mmol/L affects cell cycle progression by inducing cells to accumulate in the S phase. This effect was independent of the hMLH1, hMSH2, and p53 status because it was observed to a similar extent in all cell lines under investigation. Moreover, wash-out experiments demonstrated reversibility within 48 hours. Although p53 did not have a causative role, p53 Ser15 was strongly phosphorylated. Proteins involved in the ATM-and-Rad3-related kinase (ATR)-dependent S-phase checkpoint response (Chk1 and Rad17) were also phosphorylated but not ataxia telengectasia mutated kinase.

Conclusions— Our data demonstrate that 5-ASA causes cells to reversibly accumulate in S phase and activate an ATR-dependent checkpoint. The activation of replication checkpoint may slow down DNA replication and improve DNA replication fidelity, which increases the maintenance of genomic stability and counteracts carcinogenesis.

Patients with inflammatory bowel disease (IBD) of the colon have increased risk of developing colorectal cancer (CRC).¹ Early age at diagnosis, extent of disease, severity of inflammation, presence of primary sclerosing cholangitis, and family history of cancer have been established

Address requests for reprints to: Christoph Gasche, MD, MUW Wien, KIM4, Währinger Gürtel 18, A-1090 Vienna, Austria. e-mail: christoph.gasche@meduniwien.ac.at; fax: (43) 1-404004735..

The authors thank Ted Hupp (University of Edinburgh, United Kingdom) for the kind gift of the HCT116 ^{p53^{-/-}} cells; C. Richard Boland, MD (Baylor University Medical Center, Dallas, TX), for the kind gift of the HCT116+chr3 cells; Cornelia Lichtenberger (MUW, Vienna) for her valuable help with the flow cytometry experiments; Peter Burgers (Washington University, St. Louis, MO) for the gift of the DNA polymerase δ ; and James R.A. Hutchins (IMP, Vienna) for helping with the immunofluorescence settings and useful discussion.

Supported by the Austrian Science Fund (grants M-874-B14 to M.G.L. and P18270 to C.G.); by the International Organization of Inflammatory Bowel Diseases; and by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

as independent risk factors for the development of CRC in ulcerative colitis (UC).^{1–4} Prevention of CRC by administration of chemopreventive agents is one of the most promising options for IBD patients. The chemo-preventive efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) against intestinal tumors has been well established, but, because NSAIDs may aggravate the symptoms of colitis, their sustained use for the purpose of CRC chemoprevention has been contraindicated in IBD. A promising candidate drug for chemoprevention in IBD patients is mesalazine, (the active compound of sulfasalazine) or 5-aminosalicylic acid (5-ASA). In fact, the discontinuation of 5-ASA therapy was associated with a higher CRC risk.⁵ Successful prevention of colonic dysplasia and cancer further supports the potential role of 5-ASA as a chemopreventive agent in IBD,⁶ but the mechanism underlying these effects remains unknown.⁷ It is assumed that 5-ASA has similar genetic and molecular targets as NSAIDs, which is further supported by its structural similarity with aspirin. It was reported that 5-ASA had antiproliferative effects on colon cancer cell lines,⁸ but other studies have failed to show a significant antiproliferative effect of 5-ASA against several colon cancer cells in culture⁹ or against colon tumors in a rat model.¹⁰

Several groups found that 5-ASA significantly induced apoptosis and decreased proliferation in colorectal mucosa in patients.^{11,12} Most studies have focused on 5-ASA's anti-inflammatory properties. A few reports suggest that 5-ASA might act by blocking the transcription factor nuclear factor (NF)- κ B and/or stabilizing the inhibitor I κ B both in vivo¹³ and in mouse models.¹⁴ It has been recently reported that 5-ASA affects the Wnt/ β -catenin pathway in adenomatous polyposis coli (APC) mutated cells, by inhibiting the activity of PP2A phosphatase and therefore stimulating β -catenin degradation.¹⁵ 5-ASA has also been shown to interact with the Wnt/ β -catenin target peroxisome proliferator-activated receptor (PPAR)- γ , which is involved in inflammation-driven colon carcinogenesis.¹⁶ A recent study showed that 5-ASA treatments had a beneficial effect on colitis induced in wild-type mice but not in heterozygous PPAR- γ (+/-) mice.¹⁷ In epithelial cells, 5-ASA increased PPAR- γ expression and its activation and promoted its translocation into the nucleus, all effects independent from its weak cyclooxygenase (COX) inhibitory properties. Finally, Monteleone et al¹⁸ recently showed that 5-ASA disrupts epidermal growth factor receptor (EGFR) signaling and inhibits EGFR activation by enhancing the activity of the SH-PTP1 and SH-PTP2 phosphatases both in CRC cell lines and in ex vivo organ cultures of CRC explants.

One mechanism by which genome stability is maintained and cellular proliferation is regulated is that of activating checkpoint responses.¹⁹ Cell cycle checkpoints are activated by DNA damage and incomplete DNA replication.²⁰ If DNA synthesis is inhibited or disturbed, an S-phase checkpoint stabilizes replication components and slows down DNA synthesis.²¹ Recent lines of research suggested that 5-ASA alters cell cycle progression²²: when colon cells were exposed to high doses of 5-ASA, an increase of aberrant mitotic cells and mitotic arrest was observed, followed by cellular death.

Our group has recently determined the influence of 5-ASA on the rate of mutation in a repetitive DNA sequence in cultured colorectal cells; this compound reduces the rate of spontaneous mutations, independently of its anti-inflammatory properties.²³ We hypothesized that one key mechanism by which 5-ASA reduces the mutation rate is by interacting with cellular machineries involved in progression through the cell cycle. This would result in slowing down processes such as DNA replication (S phase) and cell division (mitosis) through the onset of cell cycle checkpoints, which would give the cell the opportunity to either repair the damage that the DNA may have encountered or undergo apoptosis. In both cases, this would prevent accumulation of mutated or damaged cells and would lead to maintenance of DNA integrity. In this study, we therefore investigated the effects of 5-ASA cell cycle progression with the aim of determining the underlying cellular mechanisms. Here, we show that 5-ASA inhibits the proliferation of colon cancer cells in a dose- and time-dependent manner and leads to the

accumulation of cells in S phase independently of both p53 and an active mismatch repair (MMR) complex. High concentrations of 5-ASA cause apoptosis and induce the appearance of abnormal mitotic cells.

Materials and Methods

Cell Lines

HT29 (mutant p53^{R273H}), LoVo (hMSH2 mutant), and HCT116 (hMLH1 mutant) colorectal cancer cells were obtained from the ATCC. HCT116^{p53-/-} cells (p53 null) were originated in Prof. Burt Vogelstein's laboratory as described.²⁴ HCT116+chr3 (hMLH1 wild-type) were a kind gift from Dr. Boland's laboratory.²⁵ Cells were grown in monolayers in Iscove's modified Dulbecco's medium (IMDM; GIBCO/Invitrogen, Lofer, Austria) containing 2 nmol/L glutamine and 10% fetal bovine serum (FBS) at 5% CO₂. The medium for HCT116+chr3 contained 400 µg/mL of G418 (GIBCO/Invitrogen).

Antibodies and Reagents

5-ASA (Sigma-Aldrich Handels GmbH, Vienna, Austria) was dissolved in culture medium at 40 mmol/L and pH adjusted to 7.2 with NaOH. Aphidicolin (Sigma), Nocodazole (Sigma), and bromodeoxyuridine (BrdU) (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and sterile filtered. Caffeine (Sigma) was dissolved in H₂O and used at 5 mmol/L. Cells were treated at subconfluent densities. All experiments were performed in triplicate. Total cell lysates were obtained by harvesting pellets of treated cells in lysis buffer (20 mmol/L Hepes, pH 7.5, 100 mmol/L NaCl, 0.2% NP-40, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT) in the presence of protease inhibitors (1 mmol/L Pefabloc [Promega GmbH, Mannheim, Germany], 1 mmol/L aprotinin, and Complete Protease Inhibitor mix [Roche]) and phosphatase inhibitors (50 mmol/L NaF, 50 mmol/L β-glycerophosphate, and 1 mmol/L Na₃VO₄ [Sigma]). Protein lysates were clarified by centrifugation at 16,000g for 15 minutes at 4°C. To obtain the DNA-bound protein fraction, after the first lyses, the nuclear pellets were resuspended in urea-based buffer (6 mol/L urea; 150 mmol/L NaCl; 0.5% NP-40), the DNA was fragmented by sonication, and all cellular debris was precipitated by centrifugation (16,000 rpm/20 minutes at 4°C). Cell lysates and DNA-bound protein fractions were snap frozen in liquid N₂ and kept at -80°C until use. Western blots were performed according to standard procedures. Briefly, equal amounts of proteins (50–150 µg) were denatured in SDS-sample buffer and resolved on NuPAGE Tris-Acetate 4%–12% gradient gels (Invitrogen). Proteins were transferred onto a Hybond-P (Amersham Pharmacia Biotech, Uppsala, Sweden) nitrocellulose membrane in Tris-Glycine buffer and 20% methanol for 3 hours. Membranes were blocked in phosphate-buffered saline (PBS) and 5% nonfat milk for 1 hour, incubated with the appropriate primary antibody according to the manufacturer's protocol, followed by the specific horseradish peroxidase (HRP)-conjugated secondary antibody. Chemoluminescent signals were detected using ECL reagents (Amersham). All antibodies for immunoblotting were diluted in PBS, 3% BSA, and 0.03% NaN₃. Antibodies used were as follows: mouse monoclonal antibody (mAb) anti-p53 DO7 (Calbiochem); rabbit polyclonal antibody (pAb) antiphospho-p53 Ser15 (Cell Signaling Technology, Danvers, MA); mAb anti-p21waf1/cip1 (Cell Signaling); pAb for hMSH2 and hMLH1 (Pharmingen/Becton-Dickinson, Cowley, Oxford, UK); pAb antitubulin (Abcam, Cambridge, UK); pAb anti-ATR; mAb anti-MCM2; pAb anti-RPA; pAb anti-Cdc45; pAb anti-γH2AX and pAb anti-phospho-γH2AX Ser39; pAb anti-Clasp; pAb anti-Chk1 and pAb anti-phospho-Chk1 Ser317 and Ser345 (Cell Signaling); and pAb anti-Rad17 and pAb anti-phospho-Rad17 Ser645 (Cell Signaling). Yeast DNA polymerase δ²⁶ was kindly provided by P. M. Burgers.

MTT Assay

Cells were plated in 96-well microtiter plates at a density of 5000 cells/well in medium. Twenty-four hours later, cells were treated with 5-ASA (0–20 mmol/L). After treatment, cells were incubated for 3 hours at 37°C with a solution of MTT (Sigma) at a concentration of 50 mg/100 mL. The cells were lysed in 10% SDS and 0.01 N HCl for 12 hours. For each sample, the absorbance of the reduced intracellular formazan product was read at 570 nm in a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). Each assay was performed in triplicate.

Flow Cytometry Analysis

Cells were harvested, washed in PBS, and fixed in 70% ethanol for 30 minutes at room temperature; subsequently, they were centrifuged for 5 minutes at 1500 rpm, resuspended in PBS-containing propidium iodide (20 mg/mL, Sigma) and RNase (60 mg/mL, Sigma), and stained at room temperature in the dark for 15 minutes. DNA content was measured by flow cytometry, and the cell cycle distribution was analyzed using CellQuest Software (BD Technology, Mountain View, CA). For the analysis of the S-phase population, subconfluent adherent cells were pulsed with 10 μ mol/L BrdU (Sigma) for 60 minutes prior to harvesting and fixation in 70% ethanol. Cells were subsequently denatured in 2 mol/L HCl and stained with anti-BrdU monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by FITC-conjugated secondary anti-mouse IgG (Molecular Probes/Invitrogen, Lofer, Austria). After staining with propidium iodide solution, cells were analyzed by flow cytometry as described.

Immunofluorescence

Cells were seeded in 8-well chambers (Nalgene; Nalge Ltd, Hereford, UK) at 37°C to subconfluent density. Twenty-four hours later, cells were treated with 5-ASA for 24–48 hours. After treatment, cells were fixed in freshly prepared 4% paraformaldehyde (pH 7.4). Cells were permeabilized in 0.5% Triton/PBS for 10 minutes and then blocked with 3% BSA/PBS for 1 hour. After blocking, cells were incubated with mAb antitubulin (Santa Cruz) for 2 hours, to stain the mitotic spindle or the interphasic filaments, followed by FITC-conjugated secondary anti-mouse IgG (Molecular Probes) for 45 minutes. Cells were mounted onto slides in the presence of the DAPI-containing mounting medium (Vectashield, Burlingame, CA) and visualized using a Carl Zeiss Axio-plan 2 fluorescence microscope. Images were captured with a Photometrics CoolSNAP fx digital camera (Roper Scientific, Tuscon, AZ) and processed using MetaMorph (Molecular Devices, Downingtown, PA) and Canvas X (ACD Systems, British Columbia, Canada) software.

Measure of Polymerase Replication Fidelity

The fidelity of polymerase (Pol) δ was measured using an in vitro DNA synthesis assay with an M13mp2-derived gapped substrate that contained the lacZ α gene in the gapped region, as previously described.²⁷ The template contained a run of 7 template T residues in the +1 reading frame that yields colorless M23 plaques. Single-base deletion errors by Pol δ restore the correct reading frame and yield dark blue M13 plaques. To fill the gap, Pol δ was incubated with gapped M13mp2 molecules in a reaction mixture containing 40 mmol/L Tris-HCl, pH 7.8, and 8 mmol/L magnesium acetate and each of the 4 dNTPs at 250 μ mol/L. Reactions also contained either 0.1 mmol/L, 1 mmol/L, or 10 mmol/L 5-ASA or solvent alone (4% DMSO) as a control. Reaction mixtures were incubated at 30°C for 60 minutes. Gap filling was confirmed by gel electrophoresis,²⁷ and aliquots of reaction products were introduced into the MC1061 mutS strain of *Escherichia coli* by electroporation, and samples were plated along with the CSH50 complementation strain on minimal media containing IPTG and X-Gal.²⁷ The reversion frequency was calculated from the ratio of revertant (blue) plaques to the total number of plaques scored.

Statistics

Changes in the cell cycle distribution were measured by parent *t* test. A *P* value of $<.001$ was regarded as significant. Experiments were repeated at least 3 times.

Results

5-ASA Reduces the Proliferation of Human Colon Cancer Cell Lines

The effect of 5-ASA on cellular viability and cellular growth was evaluated by an assay based on the activity of mitochondrial dehydrogenase, which cleaves a soluble yellow tetrazolium salt (MTT) to blue formazan crystals, a conversion that occurs only in living cells. HCT116, LoVo, and HT29 cells were treated with 5-ASA (0–20 mmol/L) for 48 hours. 5-ASA treatment induced a significant concentration-dependent reduction in the proliferation of all cell lines (Figure 1).

5-ASA Affects Cell Cycle Progression in CRC Cells

We aimed to establish whether the reduction in growth in CRC cells was due to 5-ASA-induced changes in cell cycle progression. HT29, HCT116, and LoVo cells were cultured in the presence of 0–20 mmol/L ASA for 48 hours, and the cell cycle distribution was analyzed by flow cytometry (Figure 2A). Upon treatment with 10–20 mmol/L 5-ASA, the cell cycle profile of all cell lines changed within 48 hours, with an apparent increase in the number of cells in S phase. The best effect on cell proliferation arrest was observed at 20 mmol/L 5-ASA. Higher 5-ASA concentrations (above 20 mmol/L) affected cell survival (data not shown).

5-ASA Increases the S-Phase Population in CRC Cells

The cell cycle distribution that we observed in colon cells upon treatment with 5-ASA would be consistent with an increase in the proportion of replicating cells (S phase), suggesting that DNA replication is slowed down in these cells. Fluorescent detection of BrdU incorporation into newly synthesized DNA provides an exact measure of the number of cells in S phase. To verify our observation, we pulsed 5-ASA-treated cells with BrdU for 1 hour and measured the number of actively replicating cells by flow cytometry analysis. After 48 hours exposure to 5-ASA, both HCT116 (Figure 2B) and HT29 (Figure 2C) showed a significant increase in the number of cells in S phase when compared with the nontreated population (percentage S phase-HCT116: 0 mmol/L: $47.8\% \pm 2.9\%$; 10 mmol/L: $62.6\% \pm 2.8\%$; HT29: 0 mmol/L: $42.2\% \pm 3.6\%$; 10 mmol/L: $59.1\% \pm 3.8\%$, $P <.0001$). Moreover, prolonged treatment with low doses of 5-ASA (1.25–2.5 mmol/L) for 120 hours (Figure 2D) caused a similar S-phase arrest (120 hours: percentage S-phase-HCT116: 0 mmol/L: $46.9\% \pm 1.8\%$; 2.5 mmol/L: $61.1\% \pm 0.3\%$, $P <.0001$), suggesting that the increase in the replicating population upon 5-ASA-treatment is dose and time dependent.

5-ASA-Induced S-Phase Accumulation Is MMR-Independent

The DNA MMR system repairs base mismatches after DNA replication, inhibits recombination between nonidentical DNA sequences, and provokes both checkpoint and apoptotic responses following certain types of DNA damage. It was recently demonstrated that aspirin alters expression of MMR proteins and induces apoptosis through COX- and p53-independent mechanisms.²⁸ MMR-deficient cells are resistant to many chemotherapeutic agents and are capable of bypassing the S and G2/M checkpoint in vitro.²⁹ We therefore investigated whether MMR-deficient and -proficient colonic cells respond differently to 5-ASA-treatment: hMLH1-mutant HCT116 cells and the nearly isogenic HCT116+chr3 cells³⁰ (in which an additional copy of chromosome 3, bearing the hMLH1 wild-type gene, has been reintroduced; Figure 3A) were exposed to 5-ASA for 48 hours (Figure 3C, upper and middle panels, respectively). Both cell lines showed a significant increase in the S-phase population, with no considerable

difference between the MLH1-expressing and the MLH1-mutant cell line (percentage S phase-HCT116+chr3: 0 mmol/L: $50.8\% \pm 1.1\%$; 10 mmol/L: $63.8\% \pm 2.6\%$, $P < .0001$). This suggests that hMHL1 does not have a significant role in tuning the response to 5-ASA.

5-ASA-Induced S-Phase Accumulation Is p53-Independent

DNA damage leads primarily to G1 arrest in p53 wild-type cells, whereas p53-defective tumor cells preferentially arrest in S phase or G2/M.³¹ In our study, colon cells expressing either wild-type p53 (HCT116) or a mutant form of the tumor suppressor protein (HT29) responded similarly to 5-ASA treatment by arresting in S phase. It has been reported that the presence of mutations in the p53 gene does not affect DNA replication fidelity or DNA repair.³² However, other experimental evidence suggested that p53 is required for the lasting maintenance of the replication checkpoint.³³ We used HCT116^{p53-/-}, a p53-null HCT116-derived cell line²⁴ (Figure 3B), to test whether p53 is required for the 5-ASA-induced S-phase accumulation (Figure 3C, lower panels). Upon treatment with 5-ASA, p53-null cells showed a replication arrest very comparable with that of the p53 wild-type population (percentage S phase-HCT116^{p53-/-}: 0 mmol/L: $47.4\% \pm 2.9\%$; 10 mmol/L: $59.7\% \pm 2.1\%$, $P < .0001$), indicating that p53 does not have a causative role in the 5-ASA-mediated cell cycle arrest.

Checkpoint Kinase Chk1 and Checkpoint-Dependent Clamp-Loader Rad17 Are Activated Upon 5-ASA Treatment

The DNA damage/replication checkpoints act by sensing the presence of damaged DNA or of stalled replication forks and initiate signaling pathways that arrest cell cycle progression. Recent studies have demonstrated that activation of the replication checkpoint in higher eukaryotes is dependent on the phosphorylation of the Chk1 kinase by the PI-3 like kinases ataxia telangiectasia mutated kinase (ATM) and ATM-and-Rad3-related kinase (ATR).³⁴ The replication factor C (RFC)- and PCNA-related checkpoint protein Rad17 shares regions of homology with the 5 subunits of RFC and is essential for the checkpoint-dependent phosphorylation and activation of Chk1 when the replication checkpoint is induced.³⁵ We found that the well-characterized ATR target site Chk1 Ser317 was strongly phosphorylated upon 8 hours exposure to 5-ASA in both HCT116 and HT29 cells (Figure 4A). In a time-course experiment, HCT116 cells were exposed to 0, 20, or 40 mmol/L 5-ASA for 2–8 hours. Analysis of total cell lysates indicated a strong phosphorylation of Chk1 at Ser345 and of Rad17 at site Ser645 as early as 2 hours after treatment (Figure 4B).

5-ASA also induced phosphorylation of p53 on Ser15 in both cell lines (Figure 4B). Phosphorylation of p53 on Ser15 by ATM and ATR triggers posttranslational modifications, which contribute to p53 stabilization and activation.³⁶ In HCT116 cells, Ser15 phosphorylation correlated with a significant increase in the levels of the major p53-target factor, p21^{waf1/cip1}, which was time and concentration dependent. Thus, although p53 was found not to have a causative role in the S-phase arrest in our system, the p53 pathway seemed to be activated following exposure to 5-ASA.

Replication Checkpoint Sensors ATR and Claspin Are Activated and Recruited Onto The DNA Upon Treatment With 5-ASA

In mammalian cells, the DNA replication checkpoint is dependent on ATR. Following replication fork stalling, ATR associates with the ATR-interacting protein (ATRIP) and localizes to intranuclear foci of arrest of DNA synthesis. Upon completion of replication or release of the replication checkpoint,^{37,38} it dissociates from the chromatin. Therefore, its accumulation onto the chromatin indicates inhibition or stalling of DNA replication. An additional factor involved in the DNA damage/replication checkpoint in mammalian cells is claspin, a recently identified protein that regulates Chk1 activation. Claspin is a cell cycle regulated protein that localizes onto the DNA and associates with Chk1 following replication

stress upon phosphorylation by ATR.³⁹ To test further whether cells responded to 5-ASA treatment by activation of a DNA replication checkpoint, we analyzed the chromatin-bound protein fraction in HCT116 and HT29 cells exposed to 5-ASA for 2–8 hours (Figure 5B and data not shown). We found that both ATR and claspin associated with the DNA within 2 hours of 5-ASA treatment and remained bound for up to 8 hours, suggesting the induction of an ATR- and claspin-dependent S-phase arrest in colon cells. In contrast, no activation of the ATM kinase by autophosphorylation at Ser1981 was detectable in HCT116 cells upon treatment with 5-ASA (data not shown), which is consistent with the 5-ASA activating an ATR-dependent/ATM-independent checkpoint. No significant levels of ATR or claspin were recruited to the chromatin in untreated HCT116 between 2 and 24 hours (Figure 5A).

Treatment With 5-ASA Causes Accumulation of Replication Proteins MCM, Cdc45, and RPA Onto DNA Origins

The sequential, cell cycle-dependent assembly of the origin replication complex, CDC6 protein, and the minichromosome maintenance (MCM)2–7 helicases (which all together take the name of prereplication complex) at origins of replication ensures that DNA replicates only once per cell cycle.⁴⁰ The periodic association of the MCM complex with chromatin is regulated via phosphorylation by cyclin-dependent kinases.⁴¹ Activation of the S-phase checkpoint, while stabilizing the binding of the MCM complex and Cdc45 with the stalled origins of replication, inhibits association of Cdc45 with late-firing origins.⁴² The binding of claspin to chromatin depends on the pre-replication complex and Cdc45, whereas RPA is required for the activation of both ATR and Rad17.⁴³ When the DNA-bound fractions of treated cells were analyzed (Figure 5B), we found that the MCM complex and the replication proteins Cdc45 and RPA were stably associated with the chromatin and accumulated onto the DNA for at least 8 hours, further suggesting that the normal timing of DNA replication had been altered and slowed down upon 5-ASA treatment. Moreover, γ H2AX, a member of the histone H2A family, was rapidly phosphorylated at site Ser139, which has been reported to be an ATR target following DNA-replication stress.⁴⁴ All these pieces of evidence point to the prompt activation of a replication checkpoint in colon cells, in response to 5-ASA, which may be slowing down the rate of DNA synthesis.

Cells That Escape the Replication Checkpoint Undergo Abnormal Mitotic Cell Division Upon 5-ASA Treatment

The S-phase checkpoint, and associated decrease in the rate of DNA synthesis, is known to have a transient activity.^{45,46} A conflict in cell cycle progression or DNA damage can lead to mitotic catastrophe when the DNA checkpoints are inactivated. Mitotic catastrophe is a particular type of cell death that occurs during mitosis and results from a combination of DNA damage and leaky cell cycle checkpoints. Failure to arrest the cell cycle before or at mitosis triggers an attempt of aberrant chromosome segregation, which culminates in the generation of aneuploid cells and, ultimately, in the activation of the apoptotic pathway.^{47,48} When 5-ASA-treated HT29 cells were stained for the spindle component tubulin, we observed a number of mitotic cells, which presented aberrant spindle structures (Figure 6A and B). These abnormal structures included tripole and “weak” spindles and acentric pole bodies and appeared at sublethal concentrations of 5-ASA (20 mmol/L), after installing of the replication checkpoint (48 hours), whereas no aberrant mitotic cells were visible in the control or at 24 hours in treated cells, before the S-phase arrest was detected, even at high doses of 5-ASA (data not shown). We suggest that these aberrant mitotic cells derived from cells in which the DNA synthesis was stalled by 5-ASA but, somehow, escaped the replication checkpoint and tried to divide with an unreplicated set of chromosomes. Cells showing features of mitotic catastrophe, such as giant or multi-lobular nuclei,⁴⁷ were also visible in HCT116 (Figure 6C).

5-ASA-Induced Replication Checkpoint Is Reversible Upon Treatment Release

Activation and phosphorylation of the checkpoint kinase ATR and Chk1, as well as p53 and Rad17 phosphorylation, may be associated with cellular responses to replication defects and/or DNA damage. To verify that no permanent damage was caused to the treated colon cancer cells, we exposed HCT116 cells to 0 or 10 mmol/L 5-ASA for 48 hours, followed by incubation of these cells under normal culture conditions for a further 48 hours (Figure 7). When treated cells were reversed to normal conditions, their profile was similar to that of untreated cells (percentage S phase 0 mmol/L, 96 hours: $46.2\% \pm 0.3\%$; 10 mmol/L, 48 hours then 0 mmol/L, 48 hours: $56.2\% \pm 2.4\%$, $P < .0001$). In contrast, cells exposed to 5-ASA for 96 hours maintained a significant S-phase arrest (10 mmol/L, 96 hours: $69.1\% \pm 0.4\%$). This suggests that replication checkpoint and other cellular changes induced by 5-ASA treatment are reversible and that chronic exposure to 5-ASA is required to ensure slowing down of the replication machinery and accumulation of cells in S phase.

5-ASA Does Not Affect Replication Fidelity of DNA Polymerase δ

DNA Pol δ is one of 3 polymerases required for eukaryotic DNA replication and is believed to be responsible for copying a large part of genomic DNA.⁴⁹ To examine whether 5-ASA directly influences Pol δ fidelity when replicating a repetitive DNA sequence, we assessed the accuracy of DNA synthesis by yeast Pol δ when copying a homonucleotide run sequence of 7 consecutive template Ts. Deletion of a single T nucleotide in the run by Pol δ restores the normal *lacZa* reading frame, resulting in a revertant blue plaque phenotype. The reversion frequency for single nucleotide deletions in the 7-T run was determined for Pol δ in the presence of 0.1 mmol/L, 1 mmol/L, or 10 mmol/L 5-ASA (Table 1). Reversion frequencies were indifferent to the presence of 5-ASA (Table 1), indicating that 5-ASA does not directly affect Pol δ fidelity for single-base deletions in this microsatellite sequence.

Discussion

The convincing clinical evidence for chemopreventive effect of 5-ASA in patients with IBD has prompted studies of its molecular mechanisms of action. It is generally believed that 5-ASA has specific activities other than anti-inflammatory or oxygen scavenging that interferes with carcinogenesis.

Our group has previously shown that 5-ASA decreases the rate of spontaneous mutations in colon cancer cells, which was independent from the presence of a functional mismatch repair complex,²³ thus suggesting that 5-ASA enhances replication fidelity by interacting directly with the replication enzymes. Our results further support this observation and illustrate that 5-ASA not only affects cell proliferation and induces apoptosis in all colon cell lines tested but also activates a specific replication checkpoint, which leads to accumulation of cells in S phase and to a reduction of the rate of DNA synthesis (see Figure 8 for a summarizing model).

If DNA synthesis is inhibited or disturbed, the intra-S-phase checkpoint stabilizes replication components and prevents initiation at late DNA origins,²¹ thus slowing down DNA synthesis and maintaining the replication fork integrity.⁵⁰ Evidence for the induction of a replication checkpoint upon treatment with 5-ASA in our system, apart from an increase in the proportion of cells in S phase (Figure 2), includes recruitment of the sensor kinase ATR and of the adaptor molecule claspin onto the chromatin fraction; phosphorylation of the downstream ATR targets Rad17 and Chk1, as well as of histone γ H2AX; and a delay in the completion of DNA synthesis, as indicated by the accumulation of replication factors MCM, Cdc45, and RPA onto DNA replication foci (Figures 4 and 5). In our system, the interference of 5-ASA with cell cycle progression is p53 independent, a factor whose activation normally leads to G1 arrest. In that, 5-ASA differs from other salicylate derivatives, such as aspirin,^{28,51} COX2 inhibitors, and

other NSAIDs,⁵² and it makes it a good candidate for chemoprevention of ulcerative colitis (UC)-derived cancers, in which p53 mutations are early events in oncogenesis.⁵³

Differently from the G1 and G2 arrest, the S-phase checkpoint does not cause a complete halt of cell proliferation but instead delays cell cycle progression through mitosis.⁵⁴ The fact that 5-ASA induces S-phase arrest is consistent with a direct interaction of 5-ASA with the replication machinery, which may lead to decreased replication errors.²³ One may speculate that slowing of replication may give more time to the replication polymerases to exert their “proof-reading” activity. However, *in vitro* results suggest that any slowing of the replication complex by 5-ASA does not directly reduce Pol γ replication fidelity when copying a microsatellite sequence (Table 1). It is of course possible that 5-ASA may alter fidelity in other sequence contexts or interfere with other factors of the replication machinery such as PCNA, RFC, helicases, and others or perturb replication fork structure.

Reinacher-Schick et al²² described an increase of aberrant mitotic cells and mitotic arrest upon treatment with high doses of 5-ASA (above 30 mmol/L) in HT29 cells. We were not able to reproduce their results; although initial analysis of the cell cycle profiles may lead to this conclusion (Figures 2 and 3), further examination of the cell cycle distribution by BrdU incorporation clearly showed no change in the G2/M-phase population. However, in HT29 cells treated with high doses of 5-ASA for 48 hours, we observed a few aberrant mitotic cells (approximately 20% of all mitotic cells), which included cells with multipolar spindles. Some of the aberrant mitotic spindles were similar to previously described “weak” spindles, depending on a defective APC pathway^{55,56} like the one in HT29 colon cells. APC plays an antagonist role in the Wnt-signalling pathway, and loss of APC function contributes to uncontrolled proliferation. Additionally, APC is involved in the regulation of cell migration, cell adhesion, chromosome segregation, spindle assembly, apoptosis, and neuronal differentiation.^{57,58} Mouse cells harboring homozygous APC mutations are frequently tetraploid.⁵⁹ In addition, colon cancer cells with APC mutations have weakened kinetochore-microtubule interactions.⁵⁸ Interestingly, aberrant mitotic cells were not visible in the APC-wild-type (and p53-wild-type) HCT116 cells. Instead, when these cells were exposed to 5-ASA, we noticed the appearance of giant polynucleated cells (Figure 6). Such abnormal cells were previously observed⁶⁰ and are consistent with the induction of mitotic catastrophe events.⁶¹ Mitotic catastrophe is a type of cell death that occurs during mitosis and results from a combination of deficient cell cycle checkpoints and cellular damage.⁴⁷ We hypothesize that few cells escape the 5-ASA-induced S-phase checkpoint and try to undergo mitosis with an unreplicated set of chromosomes. It is plausible that, in the presence of APC mutations, aberrant spindles initiate chromosome instability and aneuploidy, whereas, if the APC pathway is intact, cells suffer from defect in cytokinesis and encounter mitotic catastrophe. In both cases, however, the expected outcome would be death of genetically unstable cells.

In the treatment of UC patients, 5-ASA, given orally or topically, is rapidly absorbed. Normal daily doses range from 1 to 4 g. However, an estimate of the bioavailability in the intestinal lumen is rendered difficult by the rapid metabolism of 5-ASA to its acetylated metabolite N-acetyl-5-aminosalicylate. The potential clinical usefulness of our data is further strengthened by the intraluminal 5-ASA concentrations in UC that were estimated well above our experimental concentrations (ie, 12.6 to 23.7 mmol/L).⁶² Direct measurements of 5-ASA in the ileal fluid or in ileostomies demonstrated concentrations above 1 mmol/L.⁶³ Because 90% of the oral dose is delivered to the colon, extrapolation of these ileal measurements reveals a colonic 5-ASA concentration above 10 mmol/L, which is clearly within the concentration range used in our experiments.⁶⁴ Moreover, a study in healthy children⁶⁵ revealed that the concentration of 5-ASA in the distal gut lumen after 6 days treatment (4.44 – 6.25 mmol/L) was in a range similar to the one we used in our study.

We showed a direct time- and dose-dependent effect of 5-ASA on cultured colon cells: high concentrations are able to induce a rapid activation of a replication checkpoint: 20 – 40 mmol/L 5-ASA induces phosphorylation of the Chk1 and Rad17 effectors within 2– 4 hours (Figure 4), whereas the same concentrations lead to cell cycle arrest and subsequent cell death within 48 hours (Figure 2A and C). However, low concentrations of 5-ASA (1.25–2.5 mmol/L), which are almost completely ineffective in a short time scale (data not shown), are able to induce a similar replication checkpoint at later points in time (Figure 2D). Furthermore, the cell cycle arrest induced by 5-ASA was almost completely reversible, suggesting that no permanent damage to the cellular machinery was caused and that a continuous treatment is required for 5-ASA to exert its antiproliferative and chemoprevention effects. Our long-term results are also reassuring that 5-ASA is safe as a chemopreventive agent in IBD.

In our report, we propose a novel mechanism by which 5-ASA is able to restrain colon cell growth and proliferation while acting on the replication machinery and ensuring genome integrity, a mechanism that has not been reported for similar drugs so far. Our interpretation fits with what has been previously reported by Reinacher-Schick et al²² and provides a plausible explanation for the MMR-independent reduction of mutation rates observed by our group previously.²³ As the control of cell cycle and mutation rate is fundamental to carcinogenesis, both the activation of a replication checkpoint and the reduction of mutation rate by 5-ASA are central in the prevention of UC-related CRC. Other 5-ASA effects,^{15,17,18,22} although interesting, seem to be more secondary events. Of course, the anti-inflammatory properties of 5-ASA are upstream of such events because inflammation is considered to drive cancer development in the setting of IBD.

References

1. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G7–G17. [PubMed: 15194558]
2. Ekblom A, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer. A population-based study *N Engl J Med* 1990;323:1228–1233.
3. Askling J, Dickman PW, Karlen P, Brostrom O, Lapidus A, Lofberg R, Ekblom A. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 2001;120:1356–1362. [PubMed: 11313305]
4. Rutter M, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I, Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004;126:451–459. [PubMed: 14762782]
5. Eaden J, Abrams K, Ekblom A, Jackson E, Mayberry J. Colorectal cancer prevention in ulcerative colitis: a case-control study. *Aliment Pharmacol Ther* 2000;14:145–153. [PubMed: 10651654]
6. Rubin DTDA, Huo DZ, Yadron N, Hanauer SB. Use of 5-ASA is associated with decreased risk of dysplasia and colon cancer in ulcerative colitis. *Gastroenterology* 2003;124:A36.
7. Croog VJ, Ullman TA, Itzkowitz SH. Chemoprevention of colorectal cancer in ulcerative colitis. *Int J Colorectal Dis* 2003;18:392–400. [PubMed: 12904996]
8. Allgayer H. Review article: mechanisms of action of mesalazine in preventing colorectal carcinoma in inflammatory bowel disease. *Aliment Pharmacol Ther* 2003;18:10–14. [PubMed: 12950415]Suppl 2
9. MacGregor DJ, Kim YS, Sleisenger MH, Johnson LK. Chemoprevention of colon cancer carcinogenesis by balsalazide: inhibition of azoxymethane-induced aberrant crypt formation in the rat colon and intestinal tumor formation in the B6-Min/+ mouse. *Int J Oncol* 2000;17:173–179. [PubMed: 10853036]
10. Millar AD, Rampton DS, Chander CL, Claxson AW, Blades S, Coumbe A, Panetta J, Morris CJ, Blake DR. Evaluating the anti-oxidant potential of new treatments for inflammatory bowel disease using a rat model of colitis. *Gut* 1996;39:407–415. [PubMed: 8949646]

11. Reinacher-Schick A, Seidensticker F, Petrasch S, Reiser M, Philippou S, Theegarten D, Freitag G, Schmiegel W. Mesalazine changes apoptosis and proliferation in normal mucosa of patients with sporadic polyps of the large bowel. *Endoscopy* 2000;32:245–254. [PubMed: 10718391]
12. Bus PJ, Nagtegaal ID, Verspaget HW, Lamers CB, Geldof H, Van Krieken JH, Griffioen G. Mesalazine-induced apoptosis of colorectal cancer: on the verge of a new chemopreventive era? *Aliment Pharmacol Ther* 1999;13:1397–1402. [PubMed: 10571594]
13. Bantel H, Berg C, Vieth M, Stolte M, Kruis W, Schulze-Osthoff K. Mesalazine inhibits activation of transcription factor NF- κ B in inflamed mucosa of patients with ulcerative colitis. *Am J Gastro-enterol* 2000;95:3452–3457.
14. Kaiser GC, Yan F, Polk DB. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor κ B activation in mouse colonocytes. *Gastroenterology* 1999;116:602–609. [PubMed: 10029619]
15. Bos CL, Diks SH, Hardwick JC, Walburg KV, Peppelenbosch MP, Richel DJ. Protein phosphatase 2A is required for mesalazine-dependent inhibition of Wnt/ β -catenin pathway activity. *Carcinogenesis* 2006;27:2371–2382. [PubMed: 16728434]
16. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD. A novel therapy for colitis utilizing PPAR- γ ligands to inhibit the epithelial inflammatory response. *J Clin Invest* 1999;104:383–389. [PubMed: 10449430]
17. Rousseaux C, Lefebvre B, Dubuquoy L, Lefebvre P, Romano O, Auwerx J, Metzger D, Wahli W, Desvergne B, Naccari GC, Chavatte P, Farce A, Bulois P, Cortot A, Colombel JF, Desreumaux P. Intestinal anti-inflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor- γ . *J Exp Med* 2005;201:1205–1215. [PubMed: 15824083]
18. Monteleone G, Franchi L, Fina D, Caruso R, Vavassori P, Monteleone I, Calabrese E, Naccari GC, Bellinva S, Testi R, Pallone F. Silencing of SH-PTP2 defines a crucial role in the inactivation of epidermal growth factor receptor by 5-aminosalicylic acid in colon cancer cells. *Cell Death Differ* 2006;13:202–211. [PubMed: 16082388]
19. Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. *Science* 1989;246:629–634. [PubMed: 2683079]
20. Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. *Nature* 2000;408:433–439. [PubMed: 11100718]
21. Dimitrova DS, Gilbert DM. Temporally coordinated assembly and disassembly of replication factories in the absence of DNA synthesis. *Nat Cell Biol* 2000;2:686–694. [PubMed: 11025658]
22. Reinacher-Schick A, Schoeneck A, Graeven U, Schwarte-Waldhoff I, Schmiegel W. Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis* 2003;24:443–451. [PubMed: 12663503]
23. Gasche C, Goel A, Natarajan L, Boland CR. Mesalazine improves replication fidelity in cultured colorectal cells. *Cancer Res* 2005;65:3993–3997. [PubMed: 15899787]
24. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998;282:1497–1501. [PubMed: 9822382]
25. Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, Boland CR. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. *Cancer Res* 1994;54:4308–4312. [PubMed: 8044777]
26. Burgers PM, Gerik KJ. Structure and processivity of two forms of *Saccharomyces cerevisiae* DNA polymerase δ . *J Biol Chem* 1998;273:19756–19762. [PubMed: 9677406]
27. Bebenek K, Kunkel TA. Analyzing fidelity of DNA polymerases. *Methods Enzymol* 1995;262:217–232. [PubMed: 8594349]
28. Goel A, Chang DK, Ricciardiello L, Gasche C, Boland CR. A novel mechanism for aspirin-mediated growth inhibition of human colon cancer cells. *Clin Cancer Res* 2003;9:383–390. [PubMed: 12538492]
29. Yan T, Schupp JE, Hwang HS, Wagner MW, Berry SE, Strickfaden S, Veigl ML, Sedwick WD, Boothman DA, Kinsella TJ. Loss of DNA mismatch repair imparts defective cdc2 signaling and G

- (2) arrest responses without altering survival after ionizing radiation. *Cancer Res* 2001;61:8290–8297. [PubMed: 11719462]
30. Chauhan DP, Yang Q, Carethers JM, Marra G, Chang CL, Chamberlain SM, Boland CR. Antisense inhibition of hMLH1 is not sufficient for loss of DNA mismatch repair function in the HCT116 +chromosome 3 cell line. *Clin Cancer Res* 2000;6:3827–3831. [PubMed: 11051225]
 31. Yao SL, Akhtar AJ, McKenna KA, Bedi GC, Sidransky D, Mabry M, Ravi R, Collector MI, Jones RJ, Sharkis SJ, Fuchs EJ, Bedi A. Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of p34^{cdc2} kinase. *Nat Med* 1996;2:1140–1143. [PubMed: 8837615]
 32. Allio T, Donner EM, Preston RJ. A comparison of the roles of p53 mutation and AraC inhibition in the enhancement of bleomycin-induced chromatid aberrations in mouse and human cells. *Mutat Res* 2000;447:227–237. [PubMed: 10751606]
 33. Montagnoli A, Tenca P, Sola F, Carpani D, Brotherton D, Albanese C, Santocanale C. Cdc7 inhibition reveals a p53-dependent replication checkpoint that is defective in cancer cells. *Cancer Res* 2004;64:7110–7116. [PubMed: 15466207]
 34. Feijoo C, Hall-Jackson C, Wu R, Jenkins D, Leitch J, Gilbert DM, Smythe C. Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. *J Cell Biol* 2001;154:913–923. [PubMed: 11535615]
 35. Post SM, Tomkinson AE, Lee EY. The human checkpoint Rad protein Rad17 is chromatin-associated throughout the cell cycle, localizes to DNA replication sites, and interacts with DNA polymerase epsilon. *Nucleic Acids Res* 2003;31:5568–5575. [PubMed: 14500819]
 36. Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev* 1997;11:3471–3481. [PubMed: 9407038]
 37. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: partners in checkpoint signaling. *Science* 2001;294:1713–1716. [PubMed: 11721054]
 38. Heffernan TP, Simpson DA, Frank AR, Heinloth AN, Paules RS, Cordeiro-Stone M, Kaufmann WK. An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol Cell Biol* 2002;22:8552–8561. [PubMed: 12446774]
 39. Chini CC, Chen J. Human claspin is required for replication checkpoint control. *J Biol Chem* 2003;278:30057–30062. [PubMed: 12766152]
 40. Hua XQH, Newport J. Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. *J Cell Biol* 1998;140:271–281. [PubMed: 9442103]
 41. Lei M, Tye BK. Initiating DNA synthesis: from recruiting to activating the MCM complex. *J Cell Sci* 2001;114:1447–1454. [PubMed: 11282021]
 42. Aparicio OM, Stout AM, Bell SP. Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proc Natl Acad Sci U S A* 1999;96:9130–9135. [PubMed: 10430907]
 43. Lee J, Kumagai A, Dunphy WG. Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. *Mol Cell* 2003;11:329–340. [PubMed: 12620222]
 44. Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem* 2001;276:47759–47762. [PubMed: 11673449]
 45. Luciani MG, Oehlmann M, Blow JJ. Characterisation of an ATR-dependent intra-S phase checkpoint that regulates the replication timing programme in *Xenopus*. *J Cell Sci* 2004;117:6019–6030. [PubMed: 15536124]
 46. Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* 2003;424:1078–1083. [PubMed: 12944972]
 47. Castedo M, Perfettini JL, Roumier T, Valent A, Raslova H, Yakushijin K, Horne D, Feunteun J, Lenoir G, Medema R, Vainchenker W, Kroemer G. Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* 2004;23:4362–4370. [PubMed: 15048075]
 48. Canman CE. Replication checkpoint: preventing mitotic catastrophe. *Curr Biol* 2001;11:R121–R124. [PubMed: 11250164]

49. Garg P, Burgers PM. DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit Rev Biochem Mol Biol* 2005;40:115–128. [PubMed: 15814431]
50. Boddy MN, Russell P. DNA replication checkpoint. *Curr Biol* 2001;11:R953–R956. [PubMed: 11728320]
51. Luciani MG, Campregher C, Gasche C. Aspirin affects cell cycle progression in colorectal cells by activating a p53-dependent and a p53-independent/p21-dependent G1 checkpoint. Manuscript submitted
52. Goldberg Y, Nassif II, Pittas A, Tsai LL, Dynlacht BD, Rigas B, Shiff SJ. The anti-proliferative effect of sulindac and sulindac sulfide on HT-29 colon cancer cells: alterations in tumor suppressor and cell cycle-regulatory proteins. *Oncogene* 1996;12:893–901. [PubMed: 8632912]
53. Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP, Shields PG, Ham AJ, Swenberg JA, Marrogi AJ, Harris CC. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res* 2000;60:3333–3337. [PubMed: 10910033]
54. Ward IM, Minn K, Chen J. UV-induced ATR activation requires replication stress. *J Biol Chem* 2004;279:9677–9680. [PubMed: 14742437]
55. Dikovskaya D, Newton IP, Nathke IS. The adenomatous polyposis coli protein is required for the formation of robust spindles formed in CSF *Xenopus* extracts. *Mol Biol Cell* 2004;15:2978–2991. [PubMed: 15075372]
56. Tighe A, Johnson VL, Albertella M, Taylor SS. Aneuploid colon cancer cells have a robust spindle checkpoint. *EMBO Rep* 2001;2:609–614. [PubMed: 11454737]
57. Draviam VM, Shapiro I, Aldridge B, Sorger PK. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *EMBO J* 2006;25:2814–2827. [PubMed: 16763565]
58. Fodde R, Kuipers J, Rosenberg C, Smits R, Kielman M, Gaspar C, van Es JH, Breukel C, Wiegant J, Giles RH, Clevers H. Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol* 2001;3:433–438. [PubMed: 11283620]
59. Andreassen A, Vikse R, Mikalsen A, Adamovic T, Steffensen IL, Hjertholm H, Levan G, Alexander J. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induces genetic changes in murine intestinal tumours and cells with ApcMin mutation. *Mutat Res* 2006;604:60–70. [PubMed: 16574467]
60. Koelink, PJ.; Mieremet-Ooms, MAC.; Griffioen, G.; Verspaget, HW. Chemopreventive mechanisms of 5-aminosalicylic acid in colorectal cancer. Falk Symposium; Berlin. 2005. Poster presentation
61. Erenpreisa J, Kalejs M, Ianzini F, Kosmacek EA, Mackey MA, Emzish D, Cragg MS, Ivanov A, Illidge TM. Segregation of genomes in polyploid tumour cells following mitotic catastrophe. *Cell Biol Int* 2005;29:1005–1011. [PubMed: 16314119]
62. Staerk Laursen L, Stokholm M, Bukhave K, Rask-Madsen J, Lauritsen K. Disposition of 5-aminosalicylic acid by olsalazine and three mesalazine preparations in patients with ulcerative colitis: comparison of intraluminal colonic concentrations, serum values, and urinary excretion. *Gut* 1990;31:1271–1276. [PubMed: 2253912]
63. Christensen LA, Fallingborg J, Abildgaard K, Jacobsen BA, Sanchez G, Hansen SH, Bondesen S, Hvidberg EF, Rasmussen SN. Topical and systemic availability of 5-aminosalicylate: comparisons of three controlled release preparations in man. *Aliment Pharmacol Ther* 1990;4:523–533. [PubMed: 2129640]
64. Layer PH, Goebell H, Keller J, Dignass A, Klotz U. Delivery and fate of oral mesalamine microgranules within the human small intestine. *Gastroenterology* 1995;108:1427–1433. [PubMed: 7729635]
65. Christensen LA, Fallingborg J, Jacobsen BA, Abildgaard K, Rasmussen HH, Rasmussen SN, Hansen SH. Bioavailability of 5-aminosalicylic acid from slow release 5-aminosalicylic acid drug and sulfasalazine in normal children. *Dig Dis Sci* 1993;38:1831–1836. [PubMed: 8104772]

Abbreviations used in this paper

5-ASA

	5-amino-salicylic acid (mesalazine)
ATM	ataxia telangiectasia mutated kinase
ATR	ATM-and-Rad3-related kinase
ATRIP	ATR-interacting protein
CDK	cyclin-dependent kinase
Chk1	checkpoint kinase 1
COX	cyclooxygenase
CRC	colorectal cancer
MCM	minichromosome maintenance
MMR	mismatch repair
NF-κB	nuclear factor κ B
NSAIDs	nonsteroidal anti-inflammatory drugs
PP2A	phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
RFC	replication factor C

Figure 1.

Dose-response effect of 5-ASA on the growth of human colon cells. HCT116 (*open bars*), HT29 (*shaded bars*), and LoVo (*solid bars*) cells were plated at a density of 10^4 cells/well in 96-well microtiter plates. Twenty-four hours after seeding, the cells were treated with 5-ASA-containing culture medium (0 to 20 mmol/L) for 48 hours. The antiproliferative effects were measured by the MTT assay. The values are indicated as relative units (RU) to the control and represent the mean of 4 separate measurements. 5-ASA caused a dose-dependent growth reduction in all colon cancer cells.

Figure 2.

Cell cycle distribution in 5-ASA-treated cells. (A) HT29, HCT116, and LoVo cell lines were treated with 5, 10, or 20 mmol/L 5-ASA for 48 hours. After staining with propidium iodide, the cellular DNA content was visualized by flow cytometry, and the cell cycle distribution was analyzed using CellQuest flow cytometry software (BD Technology). All cell lines showed changes in the cell cycle profile following 5-ASA exposure within 48 hours of treatment. In further experiments, HCT116 cells (B) and HT29 cells (C) were exposed to 5, 10, or 20 mmol/L 5-ASA for 48 hours. Following treatment, 5-ASA was removed, and the cells were pulsed with 10 μ mol/L BrdU for 1 hour. After permeabilization and DNA-antigen unmasking, samples were stained with anti-BrdU antibody to visualize replicating cells and PI to visualize the total

DNA content. Cells were analyzed by flow cytometry using CellQuest flow cytometry software (BD Technology). Both cell lines showed an increase in the percentage of cells in S phase following treatment with 5-ASA. (D) HCT116 cells were treated with low doses of 5-ASA (1.25–5 mmol/L) for 120 hours (*upper panels*) or 168 hours. After pulsing with 10 μ mol/L BrdU and staining with anti-BrdU antibody and PI, cells were analyzed by flow cytometry. At later time points, an increase in the S-phase arrested population is visible at 1.25 mmol/L 5-ASA. In the flow cytometry dot blot, the upper region (R2) contains cells in S phase, the lower left region (R3) represents cells in G1, and the lower region (R4) represents cells in G2/M. The flow cytometry profiles are representative of at least 3 independent experiments.

Figure 3.

5-ASA-induced S-phase accumulation is p53- and MMR independent. (A) HCT116, HCT116^{p53-/-} (p53-null), and HT29 were blotted for the presence or not of the p53 protein. As shown by the Western blot, no expression of p53 was detected in HCT116^{p53-/-} cells. (B) HCT116 and HCT116+chr3 were analyzed for the expression of the MMR proteins hMSH2 and hMLH1. While HCT116+chr3 expressed both MMR components, only hMSH2 was detectable in HCT116 cells. (C) HCT116, HCT116+chr3 (expressing hMLH1), and HCT116^{p53-/-} cell lines were treated with 0 or 10 mmol/L 5-ASA for 48 hours. Following treatment, 5-ASA was removed, and the cells pulsed with 10 μ mol/L BrdU for 1 hour. After permeabilization and DNA-antigen unmasking, samples were stained with anti-BrdU antibody

and PI. Cells were analyzed by flow cytometry using CellQuest flow cytometry software (BD Technology). All cell lines showed a comparable increase in the percentage of cells in S phase following treatment with 10 mmol/L 5-ASA. In the flow cytometry dot blot, the upper region (*R2*) contains cells in S phase, the lower left region (*R3*) represents cells in G1, and the lower right region (*R4*) represents cells in G2/M. The flow cytometry profiles are representative of at least 3 independent experiments.

Figure 4.

5-ASA induces a replication checkpoint by inducing phosphorylation of checkpoint proteins Chk1 and Rad17 and activates the p53-dependent pathway. (A) Phosphorylation status of Chk1 at Ser317 was analyzed in total lysates of HCT116 and HT29 cells treated with 0, 20, or 40 mmol/L 5-ASA for 8 hours. The checkpoint kinase resulted strongly phosphorylated at the ATR-targeted residue 317 in both cell lines following treatment, consistent with the activation of a replication checkpoint in these cells. An antibody anti- α -tubulin was used as loading control to show an equal amount of proteins in our Western blot. (B) HCT116 cells were treated with 0, 20, or 40 mmol/L 5-ASA for 2–8 hours. One hundred micrograms of total lysates were analyzed by Western blot for the phosphorylation status of p53 Ser15, Chk1 Ser354, and Rad17 Ser645, as well as for the total levels of p21^{waf1/cip1}. All checkpoint proteins tested were phosphorylated within 2 hours of 5-ASA treatment, and phosphorylation correlated with increased expression of the CDK-inhibitor p21. An antibody anti- α -tubulin was used as loading control to show an equal amount of proteins in our Western blot.

Figure 5.

Checkpoint sensors ATR and claspin and replication proteins cdc45, MCMs, and RPA are recruited onto the DNA upon treatment with 5-ASA. (A) Chromatin-bound fractions of untreated HCT116 were analyzed at time points 0, 2, 4, 8, 16, and 24 hours for the presence of replication proteins recruited onto the DNA. No significant difference in the loading of ATR or claspin was detected in our samples. (B) The DNA-bound protein fraction of HCT116 cells treated with 20 or 40 mmol/L 5-ASA for 2–8 hours was analyzed by Western blot for the presence of checkpoint sensor proteins ATR and Claspin, as well as the replication proteins MCM2, cdc45, and RPA. Following exposure to 5-ASA, ATR, claspin, cdc45, and RPA accumulated onto origins of DNA within 2 hours, whereas levels of MCM2, a component of

the helicase MCM2-7 complex, increased onto the chromatin after 8 hours, suggesting a reduction in the DNA replication rate in treated cells. Accumulation of checkpoint proteins onto origins of replication correlated with increased levels of phosphorylation of the γ H2AX at Ser139 (*bottom panel*), consistent with the activation of an ATR-dependent replication checkpoint. Total levels of the histone H3 are also shown as loading control.

Figure 6.

Colon cells present aberrant mitotic cells upon 5-ASA-treatment. HT29 (A and B) and HCT116 (C) cells were exposed to 20 mmol/L 5-ASA for 48 hours. Cells were fixed in 4% PFA, permeabilized, and incubated with mAb antitubulin followed by FITC-conjugated secondary anti-mouse IgG. Cells were visualized using a Carl Zeiss Axioplan 2 fluorescence microscope, and images were captured with a Photometrics Coolsnap fx digital camera (Roper Scientific) and processed using MetaMorph (Molecular Devices) and Canvas X (ACD Systems) software (original magnification, $\times 300$). Arrows show aberrant mitotic cells (HT29; A and B) or giant cells with multilobular nuclei (HCT116; C) that appeared upon 48-hour treatment with 5-ASA.

Figure 7.

5-ASA-dependent S-phase arrest is reversible in colorectal cancer cells. HCT116 cells were treated with 0 or 10 mmol/L 5-ASA for 48 hours. Viable cells were either reverted to normal medium or kept in 10 mmol/L 5-ASA for an additional 48 hours. Cells were pulse labelled with 10 μ mol/L BrdU, harvested, and the BrdU-positive cells, as well as the total DNA content, analyzed upon anti-BrdU and propidium iodide staining (PI) by flow cytometry. The S-phase arrest observed at 10 mmol/L 5-ASA was almost completely reversible upon drug removal, indicating that this concentration of 5-ASA does not cause permanent cell damage. In the flow cytometry dot blot, the upper region (R2) contains cells in S phase, the lower left region (R3) represents cells in G1, and the lower right region (R4) represents cells in G2/M. The flow cytometry profiles are representative of at least 3 independent experiments.

Figure 8.

Model of activation of the 5-ASA-induced checkpoint responses in colon cells. Following environmental signals, 5-ASA may synergize with checkpoint sensor proteins upstream of an ATR-dependent replication checkpoint pathway. ATR, activated by association with ATRIP and recruited onto DNA replication origins, phosphorylates Chk1 on Ser345 and Ser317 and Rad17 on Ser645. The loading of claspin onto foci of replication also mediates stabilization and activation of the checkpoint kinase Chk1 and mediates the activation of the S-phase arrest. The ultimate target of Chk1 that mediates the replication stalling is yet unknown. In addition, ATR phosphorylates the tumor suppressor protein p53 on Ser15, which, although not involved in the replication checkpoint, ultimately results in p53 stabilization and transcriptional activation of downstream targets such as the CDK inhibitor p21^{waf1/cip1}. Similarly, targets of the checkpoint kinase Chk1, not involved in the replication checkpoint, such as Cdc25A/C, are expected to be phosphorylated.

Table 1Fidelity of Pol δ in a 7-T Homonucleotide Run

5-ASA (mmol/L)	Plaque number		Reversion frequency ($\times 10^{-5}$)
	Revertant	Total ($\times 10^3$)	
0	120	68	180
0.1	83	69	120
1	90	54	170
5	80	48	170
10	51	24	210