

The Position of the Amino Group on the Benzene Ring Is Critical for Mesalamine's Improvement of Replication Fidelity

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Background: Individuals with ulcerative colitis are at high risk of developing colitis-associated cancer. 5-Aminosalicylate (5-ASA) protects from cancer by its antiinflammatory activity as well as by altering cell growth, inducing apoptosis, and reducing replication errors. So far neither 5-ASA's structural specificity nor its pharmacophore group have been identified. Here we compared 5-ASA with its analogs (4-ASA and 3-ASA) and its metabolite N-acetyl-5-ASA (NAc-5-ASA).

Methods: Superoxide scavenging was analyzed by lucigenin-amplified chemiluminescence. Cell growth, cell cycle distribution, and replication fidelity at a (CA)13 microsatellite were measured in HCT116 and HT29 colon epithelial cells by MTT and flow cytometry. Nuclear protein extracts were blotted for replication protein A (RPA), claspin, p53, and p53^{Ser15}.

Results: All compounds inhibited the growth of colon epithelial cells at a similar level and displayed potent scavenging properties, with 3-ASA being the most active, followed by 5-ASA, 4-ASA, and NAc-5-ASA. Besides 5-ASA, only 4-ASA caused an increase in the S-phase population (56%–69% and 49%–62% in HCT116 and HT29 cells, respectively). This was accompanied by nuclear recruitment of replication proteins RPA and claspin as well as phosphorylation of p53^{Ser15}, both of which were weaker or absent with 3-ASA or NAc-5-ASA. 5-ASA was the only compound that lowered mutations at a (CA)13 microsatellite.

Conclusions: 5-ASA shares its growth inhibitory and superoxide scavenging properties with its structural analogs and metabolite, but the position of the amino group is critical for reducing replication errors.

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Mesalamine (5-aminosalicylic acid; 5-ASA) is a compound derived from sulfasalazine,¹ a molecule that had been discovered over 50 years ago for treatment of rheumatoid arthritis and ulcerative colitis (UC).² The covalent link of sulfapyridine and salicylate through an azo-bond was designed for combining the antibacterial activity of sulfapyridine together with the antiinflammatory properties of salicylates while reducing gastric irritation.² Since then aminosalicylates became the most important drugs for UC both for induction and maintenance of remission. In recent years 5-ASA's preventive activity against colitis-associated cancer became apparent.³ The epidemiological data supporting this notion, however, are uncertain^{3–6}; a controlled clinical trial to prove or disprove 5-ASA's chemopreventive activity is difficult to design. A better understanding of the molecular chemopreventive properties of 5-ASA is an alternative approach to this critical question. Various 5-ASA preparations exist on the market that differ in drug encapsulation, which share a delay-release formulation for intestinal predominant large bowel delivery. Since most of the 5-ASA is metabolized to N-acetyl-5-ASA (NAc-5-ASA) within the intestinal mucosa and the liver, it is considered a drug acting locally with little or no systemic effects.⁷ In fact, measurement of intraluminal 5-ASA indicated high local concentrations,⁸ which may be even higher with the recently introduced once-daily dosing regimen.⁹ The structural 5-ASA analog 4-ASA (para-aminosalicylic acid) that is used as tuberculostatic agent has also been tested for the treatment of inflammatory bowel diseases (IBDs).¹⁰ 4-ASA has never made it to a broad application either in UC or Crohn's disease (CD), despite promising studies. No clinical or laboratory data exist on 3-ASA (meta-aminosalicylic acid).

The antiinflammatory properties of 5-ASA include direct scavenging of reactive oxygen species,¹¹ inhibition of inducible nitric oxide synthase (iNOS),¹² downregulation of cyclo-oxygenase (COX),¹³ activation of the nuclear factor κ B (NF- κ B) pathway,¹⁴ and antibacterial properties.¹⁵ 5-ASA was also shown to induce apoptosis in tumor cells, inactivate epidermal-growth-factor-receptor (EGFR)

signaling,¹⁶ and downregulate the Wnt/ β -catenin signaling pathway.¹⁷ Our previous research showed that 5-ASA increases replication fidelity independently of a functional mismatch repair system¹⁸ and alters cell cycle progression in the S-phase through checkpoint activation¹⁹; however, the 5-ASA molecular targets behind these properties are unknown.

One strategy to identify molecular targets of 5-ASA is to study 5-ASA's pharmacophore. The pharmacophore of a molecule is the ensemble of steric and electronic features that is necessary to ensure optimal supramolecular interactions with a specific biological target and to trigger (or to block) its biological response.²⁰ An *in silico* docking model had identified peroxisome proliferator-activated receptor- γ (PPAR- γ) as a potential target of 5-ASA.²¹ 5-ASA docks within the PPAR- γ ligand-binding via hydrogen bonds, induces conformational changes in PPAR- γ and stimulates its translocation to the nucleus.²¹ Structure-activity modeling revealed that 5-ASA fits better than 3-ASA or NAc-5-ASA into the PPAR- γ active site, which is reflected in their reduced ability to activate PPAR- γ .²² Since 5-ASA was shown to interact with several other pathways it is likely that other proteins also serve as targets.¹⁴

The aim of this study was to identify if the NH₂-group at position 5 in 5-ASA plays a role in enabling S-phase checkpoint activation and the improvement of replication fidelity. 5-ASA and its structural analogs were tested for superoxide ($\cdot\text{O}_2^-$) scavenging, growth inhibition, cell cycle arrest, and replication fidelity in colon epithelial cells. We found that all compounds were potent $\cdot\text{O}_2^-$ scavengers and inhibited cell growth, but only 5-ASA and 4-ASA were capable to activate a replication checkpoint. Interestingly, only 5-ASA improved replication fidelity, suggesting that the position of the amino group is critical for exercising this effect.

MATERIALS AND METHODS

Cell Lines

HT29 (mutant p53^{R273H}), HCT116 (mutant hMLH1), and HCT116+chr3 (hMLH1 wildtype) colorectal cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and Dr. R. Boland (Baylor Research Medical Center, Dallas, TX). HCT116-A2.1 and HCT116+chr3-A3.7 are reporter cell lines for replication errors derived from HCT116 and HCT116+chr3 cells and harbor a (CA)₁₃ microsatellite as bait for frameshift mutations.²³ Cells were grown in IMDM (Iscove's modified Dulbecco's medium; GIBCO/Invitrogen, Karlsruhe, Germany) containing 2 mM glutamine and 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) at 37°C and 5% CO₂. HCT116-A2.1 and HCT116+chr3-A3.7 cells were grown in selection medium containing 200 $\mu\text{g}/\text{mL}$ or 150 $\mu\text{g}/\text{mL}$ Hygromycin B, respectively; the medium for

HCT116+chr3 and HCT116+chr3-A3.7 cells was additionally supplemented with 400 $\mu\text{g}/\text{mL}$ G418 (Invitrogen).

Reagents

3-ASA (>97% purity), 4-ASA, 5-ASA (>99% purity; all from Sigma, Deisenhofen, Germany), and N-Ac-5-ASA (>97% purity; Dr. Falk Pharma, Freiburg, Germany) were dissolved in culture medium at 0–40 mM and pH-adjusted to 7.4 with NaOH. Lucigenin (Merck, Darmstadt, Germany) was dissolved in PBS^{-Ca/Mg} (GIBCO/Invitrogen). Aphidicolin and bromodeoxyuridine (BrdU; both from Sigma) were dissolved in dimethyl sulfoxide (DMSO; Sigma). Hydroxyurea (Sigma) was dissolved in H₂O.

Isolation and Activation of Neutrophils and Measurement of Superoxide Scavenging

Neutrophils were isolated and activated as described before.²⁴ Briefly, 1×10^6 neutrophils were activated with phorbol myristate acetate in IMDM in the presence or absence of compounds and the levels of $\cdot\text{O}_2^-$ were determined by lucigenin-enhanced chemiluminescence on a tube luminometer and expressed as relative light units (RLU). Superoxide dismutase (SOD, 1000 U/mL, Sigma), a specific scavenger for $\cdot\text{O}_2^-$, was used as positive control.

Preparation of Nuclear Cell Extracts and Western Blotting

Cells were harvested and washed with PBS followed by incubation with 100 μL of lysis buffer (20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% v/v glycerol, and cocktail of protease- and phosphatase-inhibitors) on ice for 5 minutes. After centrifugation at 1500 rpm for 5 minutes the cytoplasmic fraction was removed and the remaining pellets were rinsed twice with lysis buffer. Washed pellets were resuspended in 100 μL of nuclear lysis buffer (400 mM NaCl, 10 mM HEPES, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.1% v/v NP40, 1 mM DTT, 1.5% sodium dodecyl sulfate (SDS), and cocktail of protease- and phosphatase-inhibitors) followed by vortexing and incubation on ice for 10 minutes. After centrifugation at 15,000 rpm for 10 minutes, nuclear cell extracts were aliquoted and stored at -80°C for analysis. Western blot analysis was performed on 4%–12% NuPAGE gels (Invitrogen) as described earlier.¹⁹ All antibodies for immunoblotting were diluted in PBS, 3% bovine serum albumin (BSA) and 0.03% NaN₃. Antibodies used included: mouse monoclonal antibody (mAb) anti-p53 DO7 (Calbiochem, Vienna, Austria); rabbit polyclonal antibody (pAb) anti-phospho-p53^{Ser15} (Cell Signaling, Frankfurt am Main, Germany); pAb anti-RPA; pAb anti-claspin (all from Cell Signaling Technologies, Beverly, MA), and mAb anti-Oct1 (Becton Dickinson, San Jose, CA) as loading control for nuclear proteins.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay

HCT116 and HT29 were seeded in a 96-well plate at a concentration of 5×10^3 cells/well. On the next day the compounds (0–40 mM) were added for 48 hours. The cells were washed and incubated with 100 μ L of a 1:1 mixture of medium and MTT (5 mg/mL in PBS) for another 3 hours. The formazan crystals were solubilized in SDS and HCl at pH 3 and quantified at 470 nm on an enzyme-linked immunosorbent assay (ELISA) reader (Anthos htll, type 12500; AnthosLabtec Instruments, Wals, Austria).

BrdU Staining and Cell Cycle Analysis

BrdU staining for the quantification of the S-phase population was done as previously described.¹⁹ Briefly, cells were pulsed with BrdU (Sigma) followed by staining with propidium iodide and anti-BrdU monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by FITC-conjugated secondary antimouse IgG (Molecular Probes, Carlsbad, CA). DNA content was measured by flow cytometry on a FACScan (Becton Dickinson) and cell cycle distribution was analyzed using CellQuest Software (Becton Dickinson).

Analysis of Replication Errors at the (CA)13 Microsatellite

Analysis of replication errors was achieved as described previously.²³ Briefly, nonfluorescent HCT116-A2.1 and HCT116+chr3-A3.7 cells, bearing 1 (A2.1) or 2 (A3.7) copies of the EGFP-based frameshift mutation reporter plasmid pIRESHyg2-EGFP/CA13 were sorted on a FACS Vantage SE (Becton Dickinson) using CloneCyt Plus sorting technology (Becton Dickinson). Cells were analyzed on a FACScan (Becton Dickinson) and the cell counts were evaluated. The counts of low fluorescent M1 cells (intermediate mutants) and high fluorescent M2 cells (definitive mutants) were expressed as mutant fractions of the total cell number in culture.

Statistical Analysis

Experiments were carried out at least in triplicate and repeated twice. Data are represented as mean of obtained measures and compared by Student's *t*-test (in case of 2 groups) or 1-way analysis of variance (ANOVA) and post-hoc multiple comparisons using Tukey's honest significant difference. The influence of different concentrations of 5-ASA and its analogs on the proliferation of HCT116 and HT29 cells was analyzed by MANCOVA. *P*-values below 0.05 were considered statistically significant.

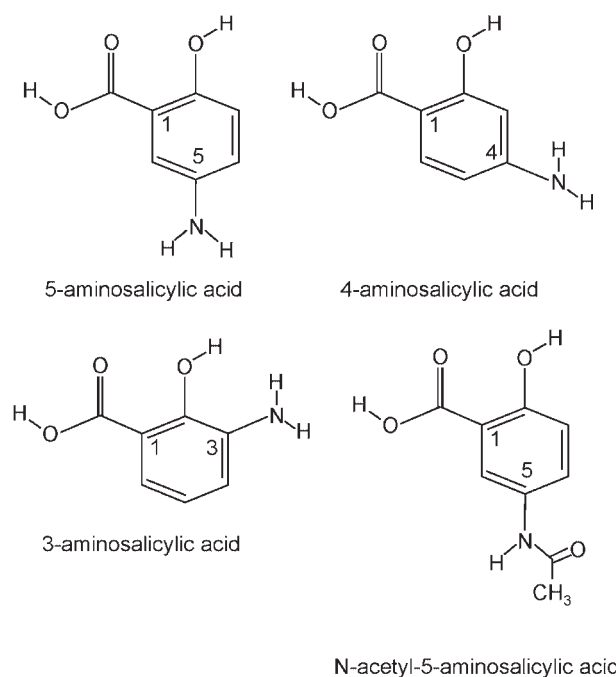


FIGURE 1. Chemical structures of 5-ASA and its analogs. 5-ASA differs from 4- and 3-aminosalicylic acid (4-ASA and 3-ASA) in the position of the amino group on the benzene ring. N-acetyl-5-aminosalicylic acid is a metabolite of 5-ASA.

RESULTS

5-ASA Analogs Reduce Proliferation of Human Colon Epithelial Cell Lines

To better understand the pharmacophore group of 5-ASA, the structural analogs 4-ASA and 3-ASA as well as the metabolite NAc-5-ASA (Fig. 1) were tested for several biological activities. Cellular proliferation was evaluated by MTT assay in HCT116 and HT29 colon epithelial cell lines. All compounds induced a dose-dependent reduction of growth rate in both cell lines ($P < 0.001$ by MANCOVA; Fig. 2). Moderate but statistically significant differences between the compounds were observed ($P < 0.001$ by MANCOVA). 5-ASA revealed the weakest inhibitory effect, whereas NAc-5-ASA was the most active inhibitor of cell proliferation in both cell lines. The IC_{50} (50% inhibition of cell proliferation) was estimated for each cell line using the regression equation for the dependency between relative units (RU) and concentration (mM). The IC_{50} for 5-ASA in HCT116 and HT29 cells was 19 mM and 24 mM, respectively, for 4-ASA 14 mM and 19 mM, for 3-ASA 15 mM and 17 mM, and for NAc-5-ASA 14 mM and 13 mM.

5-ASA Analogs Are Potent $\cdot O_2^-$ Scavengers

Reactive oxygen species released by activated neutrophils have been implicated in the oxidation of mucosal

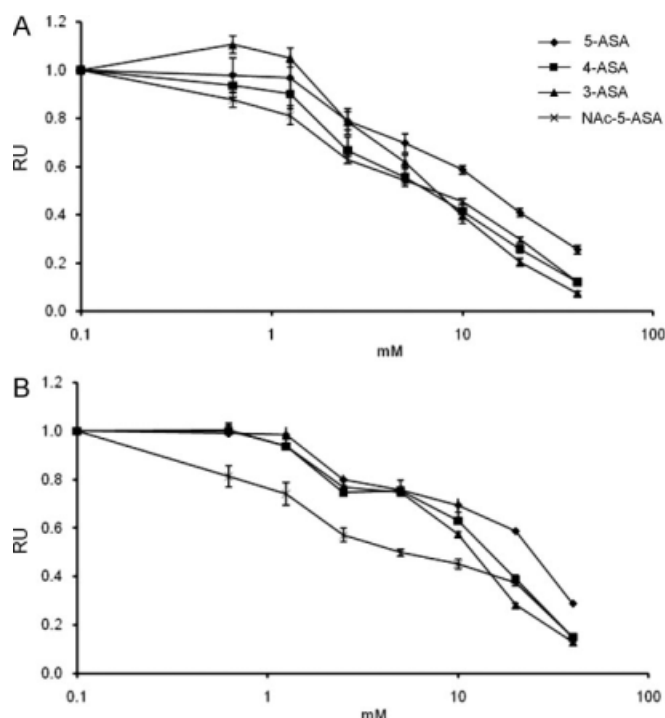


FIGURE 2. 5-ASA and its analogs interfere with proliferation in colon epithelial cells. HCT116 and HT29 cells were treated with 5-ASA and its analogs for 48 hours and analyzed by MTT assay. Percent of cell growth (percent of control in relative units; RU) was expressed as mean \pm SD value of absorbance reading from treated versus non-treated cells.

proteins²⁵ and in DNA mutations, probably causing neoplastic transformation in patients with UC.^{24,26} To test whether the 5-ASA analogs present different $\cdot\text{O}_2^-$ scavenging properties we performed a lucigenin-enhanced chemiluminescence assay. At 5 mM all compounds showed significant $\cdot\text{O}_2^-$ scavenging effects, with 3-ASA being the most active (24% of control) followed by 5-ASA (42%), 4-ASA (47%), and NAc-5-ASA (61%) (Fig. 3).

5-ASA and 4-ASA, but not 3-ASA or NAc-5-ASA, Induce an S-phase Arrest in Colon Epithelial Cells

Our previous research indicated a role for 5-ASA in the induction of a replication checkpoint.¹⁹ HCT116 and HT29 cells were treated with the 5-ASA analogs (10 mM each) for 48 hours followed by pulse-labeling with BrdU for 1 hour and cell cycle distribution was analyzed by flow cytometry. 4-ASA induced an increase in the S-phase population to a similar extent as 5-ASA, in both HCT116 (Fig. 4A) and HT29 cells (Fig. 4B). The number of replicating cells was not altered upon treatment with 3-ASA or NAc-5-ASA, indicating that this effect depends on the position and the nonacetylation of the amino group.

5-ASA Analogs Result in a Nuclear Accumulation of Replication and Cell Cycle Regulating Proteins

We previously showed that 5-ASA leads to recruitment of RPA and claspin to DNA.¹⁹ In mammalian cells, the DNA replication checkpoint depends on ATR activity. Following replication fork stalling, ATR associates with the ATR-interacting protein (ATRIP) and localizes to intranuclear foci after arrest of DNA synthesis.²⁷ Claspin is a cell cycle-regulated protein that localizes onto the DNA and associates with checkpoint kinase 1 (Chk1) upon phosphorylation by ATR,²⁸ while RPA binds to single-stranded DNA and is required for the activation of ATR.²⁹ Here we compared nuclear cell extracts after treatment with 5-ASA or analogs by Western blot followed by probing with RPA, claspin, p53, and phospho-p53 antibodies. We observed a significant nuclear increase of RPA and claspin that was fastest and strongest with 5-ASA followed by 4-ASA, 3-ASA, and NAc-5-ASA (Fig. 5). Similarly, p53 was phosphorylated after 8 hours upon treatment with 5-ASA, and later also with 4-ASA and 3-ASA but not with NAc-5-ASA. Together, these observations suggest an activation of p53 and ATR pathway proteins in response to 5-ASA but less with 4-ASA or 3-ASA.

Effect of 5-ASA Analogs on Replication Fidelity

As previously shown, 5-ASA is capable of reducing replication errors at repetitive sequences in colon epithelial cells.¹⁸ Here we compared the effect of 5-ASA analogs on the mutation rate at a (CA)₁₃ repetitive nucleotide sequence in mismatch repair deficient HCT116-A2.1 and mismatch repair proficient HCT116+chr3-A3.7 colon epithelial cells. Upon 7 days of exponential growth with

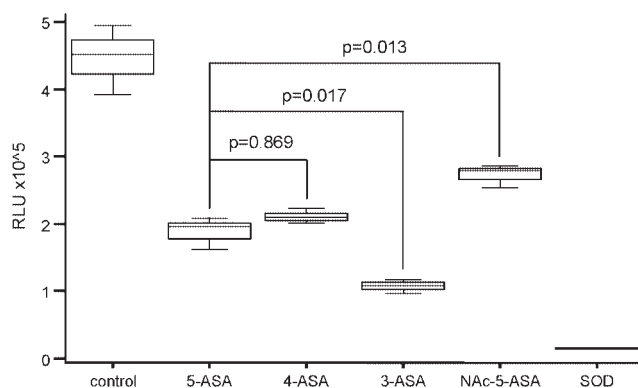


FIGURE 3. 5-ASA and its analogs are potent superoxide scavengers. 1×10^6 PMA-activated neutrophils were assayed for $\cdot\text{O}_2^-$ scavenging by lucigenin-enhanced chemiluminescence. Superoxide dismutase (SOD, 1000 U/mL, Sigma) was used as positive control. Data are represented in relative light units (RLU) as mean \pm SD and compared by 1-way ANOVA and post-hoc multiple comparisons using Tukey's honest significant difference.

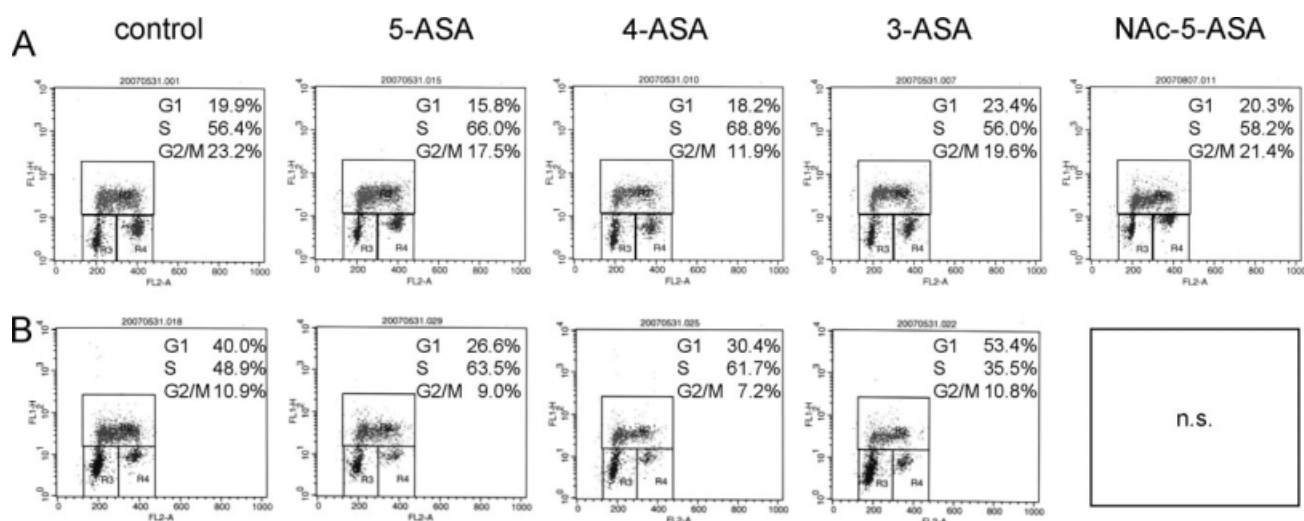


FIGURE 4. 5-ASA and 4-ASA, but not 3-ASA and NAc-5-ASA, cause an increase in the S-phase population in colon epithelial cells. Subconfluent HCT116 (A) and HT29 cells (B) were treated with 10 mM 5-ASA and its analogs for 48 hours followed by labeling with BrdU. Cell cycle distribution was analyzed by flow cytometry using CellQuest software (Becton Dickinson). Both, 5-ASA and 4-ASA caused an increase in the S-phase population (gate R2), whereas 3-ASA and NAc-5-ASA did not. NAc-5-ASA was also tested with HT29 cells in a separate experiment and showed no difference compared to control (n.s.).

compounds, the final cell number was reduced in both cell lines (Fig. 6). 5-ASA reduced cell growth in HCT116-A2.1 by 47% and in HCT116+chr3-A3.7 by 51%, 4-ASA by 83% and 82%, 3-ASA by 77% and 55%, NAc-5-ASA by 81% and 50%, respectively. Only 5-ASA and none of its analogs was capable of reducing the mutant fraction in both reporter cell lines from 2.01×10^{-3} to 1.29×10^{-3} ($P = 0.0004$) and from 1.28×10^{-3} to 5.45×10^{-4} ($P = 0.003$), respectively.

DISCUSSION

Despite its general use in the treatment of UC, the molecular actions of mesalamine are incompletely understood, specifically when it comes to its potential antineoplastic activities. The pharmacokinetics of drug delivery and metabolism implies that the point of action is located in the intestinal mucosa, more specifically, the colon epithelium. A number of various molecular mechanisms have been described in vitro and in animals.^{14–17,21,22,30–32} With an exception for PPAR- γ ,²¹ however, neither 5-ASA's structural specificity regarding the interaction with either molecular partners or 5-ASA's pharmacophore group had been identified so far. For this study we hypothesized that the amino group is part of 5-ASA's pharmacophore and therefore compared several in vitro activities with its structural analogs (4-ASA and 3-ASA) and its main metabolite (NAc-5-ASA).

Previously, we have shown a role for 5-ASA in replication control and improvement of the fidelity of DNA replication.¹⁸ We therefore compared the activity of the 5-ASA analogs on cell proliferation, $\cdot\text{O}_2^-$ scavenging, cell

cycle progression, and replication fidelity. We demonstrated that all compounds inhibited cell growth of both HCT116 and HT29 cells in a similar and dose-dependent

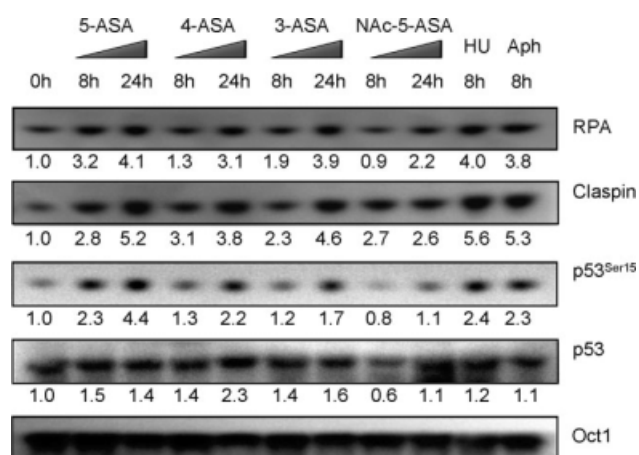


FIGURE 5. Nuclear accumulation of ATR-pathway proteins and p53 activation. Nuclear cell extracts from HCT116 cells treated with 5-ASA analogs for 8 and 24 hours were processed for Western blotting following probing with RPA, claspin, p53^{Ser15}, and p53 antibodies. Hydroxyuria (HU) or aphidicolin (Aph)-treated cells were used as controls. Scanned pictures were quantitated using TotalLab 2.0 software (Nonlinear Dynamics, UK) following normalization to Oct1 (nuclear loading control). Signal intensities were related to untreated controls (0 h). A significant increase in RPA, claspin, and p53^{Ser15} was observed upon treatment with 5-ASA, 4-ASA, and 3-ASA. This increase was already observed at 8 hours and was strongest for 5-ASA. Treatment with NAc-5-ASA revealed no changes in p53 phosphorylation.

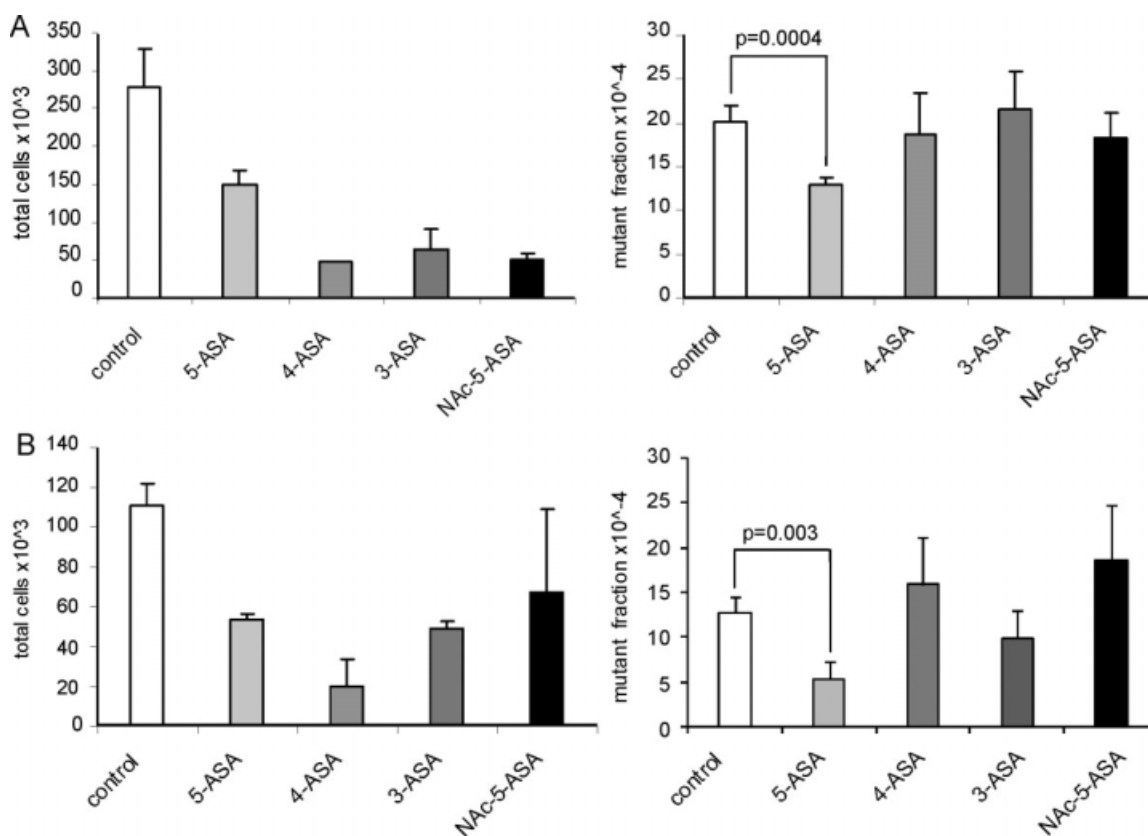


FIGURE 6. 5-ASA but none of its analogs reduces replication errors. 5-ASA analogs were tested for effects on replication errors in HCT116-A2.1 (A) or HCT116+chr3-A3.7 (B) colon epithelial cells. 1×10^3 nonfluorescent cells were sorted into 24-well dishes following incubation with 5 mM 5-ASA or its analogs for 7 days. Total cell counts and mutant fraction (MF) were analyzed by flow cytometry. 5-ASA but none of the other compounds demonstrated a significant reduction of replication errors.

fashion. Some differences among the tested compounds were observed for $\cdot\text{O}_2^-$ scavenging, with 3-ASA having the strongest effect followed by 5-, 4-, and NAc-5-ASA. Since the solubility of these compounds is comparable, the obtained differences in $\cdot\text{O}_2^-$ scavenging are likely explained by their chemical structure, among which 3- and 5-ASA are expected to be the most active scavengers: the $\cdot\text{O}_2^-$ radical should remove a hydrogen from the 5-ASA analogs and form a relatively stable intermediate. The lower the detachment energy, the easier is the hydrogen release and the stronger are the O_2^- scavenging properties. This process depends on the stability of the 5-ASA-radical, which is formed when 5-ASA is losing hydrogen. Since electronic energy of delocalization for such a radical is higher for positions 3 and 5 of the amino-group the hydrogen detachment should be facilitated for these isomers. This is not the case with 4-ASA, where hydrogen remains attached. Therefore, 3- and 5-ASA are expected to display stronger scavenging properties than 4-ASA, which was confirmed by our experiments. The chemistry of NAc-5-ASA is more complex, as 2 opposite factors should be taken into account: 1)

stronger delocalization of radical energy, and 2) less accessibility of an N-center. Theoretically this metabolite should be the weakest $\cdot\text{O}_2^-$ scavenger, which was also in line with our observations. In previous studies it was unclear whether the mutation inhibitory function of 5-ASA was related to its oxygen-scavenging properties.³³ From our results, it seems unlikely that there is a correlation between these biological properties, although 3-ASA was the best scavenger, no effect on replication fidelity was observed.

We have previously shown that 5-ASA causes cells to reversibly accumulate in S-phase by activating the ATR pathway through the checkpoint kinase Chk1 and the clamp-loader Rad17.¹⁹ The recruitment of MCM and Cdc45 to the replication fork suggested activation of a replication checkpoint that may decrease the speed of DNA replication during S-phase (i.e., replication stalling). In the current study, besides 5-ASA, only 4-ASA was able to induce accumulation of cells in S-phase. 3-ASA and NAc-5-ASA did not change the number of S-phase cells, suggesting that the 3' position or acetylation of the amino group in the 5' position abolishes this pharmacological

property of the molecule. However, the replication proteins RPA and claspin accumulated in the nucleus upon 5-ASA, followed by a lesser extent by 4-ASA, but also by 3-ASA, and very little by NAc-5-ASA. A certain level of nuclear RPA and claspin accumulation (reflecting activation of the ATR pathway) may be needed to induce effective replication stalling, a level that might not have been reached by 3-ASA and NAc-5-ASA. Similarly, activation of p53 was observed best by 5-ASA, followed by 4-ASA and 3-ASA, but not with NAc-5-ASA. Most important, however, our results demonstrate that 5-ASA but none of its analogs were capable of reducing replication errors at the (CA)₁₃ microsatellite. As 5-ASA was the only compound that increased replication fidelity together with an efficient installment of an S-phase arrest, the 5' position of the amino group seems critical for such a biological effect and thus is likely part of 5-ASA's pharmacophore, which highlights its unique biological and medical significance.

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