5-ASA - colorectal cancer - cell death : an intriguing threesome

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Chapter 4

5-Aminosalicylic acid interferes in the cell cycle of colorectal cancer cells and induces cell death modes

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Abstract

Epidemiological data suggests that 5-aminosalicylic acid (5-ASA), a non-steroidal anti-inflammatory drug used in the treatment of inflammatory bowel diseases, prevents colorectal cancer development in these patients, although the mechanisms remain incompletely understood. Here we report that 5-ASA prevents growth of several colorectal cancer cell lines by interfering in the cell cycle, i.e., an S-phase and G2/M phase arrest, dependent on 5-ASA dosage and concentration, together with an increased mitotic index. In addition, prolonged cell cycle arrest by repeated 5-ASA treatment induced apoptosis and caused abnormal spindle organization leading to mitotic catastrophe, another form of cell death. These observations illustrate that 5-ASA has chemopreventive and chemotherapeutic properties.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the Western world. With 940,000 recorded cases worldwide each year it is the third most common malignancy in the world, after lung and breast cancer, causing nearly 500,000 deaths each year worldwide [1]. Despite efforts to optimize the treatment, by surgery, radio- and chemotherapy, there is still no major improvement achieved in survival of the patients. Therefore, the prevention of CRC has become increasingly important. Independent risk factors for the development of colorectal cancer are age, alcohol use, smoking status and body mass index [2-4]. Patients suffering from chronic inflammatory bowel disease (IBD), i.e., ulcerative colitis (UC) and Crohn's disease (CD), are at increased risk for CRC development [5, 6]. 5-Aminosalicylic acid (5-ASA, mesalazine), a non-steroid-anti-inflammatory drug (NSAID) widely used in the treatment of IBD, is well tolerated, without severe side-effects and gastrointestinal toxicity [7]. Several studies, as summarized in a recent meta-analysis by Velayos *et al.* [8], suggest that the long-term use of 5-ASA in IBD patients reduces the risk of development of CRC, although some studies were not confirmative. After oral or rectal administration 5-ASA acts locally in the colon and is absorbed by colonic epithelial cells. Relatively high concentrations of intra colonic 5-ASA can be achieved of up to 50 mM, while relatively low concentration are found systemically or in the urine [9, 10]. The use of 5-ASA in rodent models of intestinal cancer has also shown that 5-ASA is able to prevent the development of intestinal tumors [11-14]. 5-ASA is therefore an attractive NSAID in a treatment and preventive strategy for CRC, although the complete mechanism has not been elucidated yet. Identifying the mechanism could lead to the rationalized application of this drug in the prevention and/or treatment of CRC. Because in cancer the delicate balance between cellular proliferation and apoptosis is deregulated, we aimed to determine the effects of 5-ASA on these features in several CRC cell lines in order to unravel possible mechanisms which might explain 5-ASA's cancer-preventive actions. 5-ASA was found to arrest CRC cells in the cell cycle and a prolonged cell cycle arrest, by repeated 5-ASA treatment, was found to induce apoptosis and mitotic catastrophe.

Material&Methods

Cell culture and materials

HT-29, HCT116 and Caco2 CRC cells were cultured in Dulbeco's Modified Eagle Medium DMEM:F12 (1:1) + GlutaMAX medium 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% (HT29 and HCT116) or 20 % (Caco2) 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 by continuous stirring, pH adjusted (~7.4) and filter-sterilized. 5-ASA enemas (Salofalk 4 gram/60 gram, Tramedico BV, Weesp, The Netherlands), were emptied and stock solutions were made similar as described above, only medium containing enema formulation could not be filter sterilized, to assess the effect of other (preserving) substances. 5-ASA incubations were refreshed every day, similar to human treatment protocols, after spinning the cells down. All experiments with 5-ASA were protected from light as much as possible. GW9662 and troglitazone (both Calbiochem, San Diego, CA, USA), were dissolved in dimethyl sulfoxide (DMSO). HT29 cells were pre-incubated for 90 minutes with GW9662 (100 nM), when indicated, before treatment with troglitazone (50 µM) or 5-ASA (30 mM) in combination with GW9662 (100 nM). DMSO was used as a control and never reached a concentration above 0.1 %.

Cell proliferation assay / cell growth analysis

For determination of proliferation cells were seeded at a density of 2 x 10^3 cells per well into 96-well flat bottom cell culture plates (Greiner bio-one, The Netherlands) and allowed to adhere for 24 hours. Cells were incubated with 100 µl of medium with or without 5-ASA for 24, 48 and 72 hours. After incubation cells were spun down at 300 g for 5 min and the incubation medium was removed. The cells were washed with PBS twice, to remove all the 5-ASA that gives background absorption at 490 nm. The AQ_{ueous} One Solution Cell Proliferation Assay was used to estimate cell viability, according to manufacturer's instructions (Promega, Madison, WI, USA). Briefly, 100 µl normal culture medium supplemented with 20 µl of MTS reagent was added and the absorbance was measured at 490 nm in a 96-well plate reader (Molecular Devices, $Thermo_{max}$ microplate reader) after incubating at 37 ˚C for 3-4 hours. The cell densities used in our experiments were in the linear range of the MTS assay. IC_{50} values were calculated [sigmoidal dose-response

(variable slope)] using Graphpad Prism (version 4.0, Graphpad Prism Inc., La Jolla, CA, USA) software. Cells were also counted by trypan blue exclusion assays using a haemocytometer, to verify results found in the MTS assays. For these experiments 2 x 10^5 cells were seeded into 6 wells plates (Corning Incorporated, Costar) and treated with 10 ml of medium with or without 5-ASA [or 2.5 ml in case of the low dosage 40 mM 5-ASA, 40 (low)], mimicking the same dosage 5-ASA/cell as in the MTS assays. Adherent cells were trypsinized and pooled with floating cells before cell countings.

Detection and quantification of apoptosis

Pooled adherent and floating cells were centrifuged onto glass slides at 350 g for 4 min (cytospins), fixed in 4 % formaldehyde in PBS (pH 7.2) and blocked with 20 % horse serum in 1% Bovine serum albumin BSA/PBS for 30 minutes at roomteperature (RT). To quantify the fraction of cells undergoing apoptosis, cytospins were incubated over night at 4°C with the M30 Cytodeath antibody (1:400 in 1% BSA/PBS, Roche Applied Science, Penzberg, Germany), that detects a neo-epitope of cytokeratin 18 (CK18) revealed after caspase-mediated cleavage. After 3 washes with PBS, the slides were incubated with biotinylated-horse-anti-mouse antibody (Vector Laboratories, 1:200 in 1% BSA/PBS) for 30 min at RT. The staining was visualized using a complex of horseradish-peroxidase (HRP) and streptavidin-ABC (Dako, Glostrup, Denmark, 1:100 in PBS), for 30 minutes at RT, and 0.05 % (w/v) diaminobenzidine (Sigma) in TRIS-HCl pH 7.6 with 0.015 % H_2O_2 for 10 min at RT, resulting in a brown staining product. Slides were counterstained with Mayer's haematoxylin (Sigma-Aldrich, Germany). The number of M30 positive cells was counted in at least 500 cells per experimental condition and expressed as percentage of apoptotic cells.

M30 and total CK18 antigen levels were detected in the culture medium by M30 and M65 ELISAs, respectively. ELISAs were preformed according to protocol by manufacturer (Peviva BV, Bromma, Sweden) and expressed as Units/L (U/L), as reported before [15].

Detection and quantification of mitosis

The number of cells with the typical morphological features of mitosis, condensed and segregating chromosomes, was counted by direct visualization. The number of mitotic cells was counted in at least 500 cells and expressed as percentage of mitotic cells, the mitotic index. Importantly, binuclear cells were counted separately, so those are not included in the mitotic index.

Immunofluerescent imaging of the mitotic spindle

HT29 cells grown on coverslips, to maintain cytoskeleton structural organization, were fixed in 4 % formaldehyde in PBS (pH 7.2), permeabilized with 0.5 % Triton X100 in PBS for 5 min, blocked with 5 % goat serum (Dako) in 1% BSA/PBS for 30 min at RT and incubated with an anti-α-tubulin antibody (Sigma, 1:1000 in 1% BSA/PBS) for 2 hours at RT. After subsequent washings coverslips were incubated with secondary goat-anti-mouse-FITC antibody (Sigma, 1:400 in 1% BSA/PBS) for 1 hour at RT. Chromosomes were stained with bis-benzimide (Hoechst 33342, Sigma) for 8 min in the dark at RT and sealed with mowoil. Cells were visualized using a Nikon Eclipse E800 microscope using filters and photographed by Nikon DXM 200 digital camera and filters. Photomicrographs were merged with Adobe Photoshop 6.0 software.

Protein lysates and immunoblot analysis

HT29 cells were trypsinized and resuspended in 1 % Triton X100/TrisHCl. Cell suspensions were sonificated 2 times for 15 seconds with a digital sonifier (Branson, model 250-D) and the cellular fragments were removed by centrifugation. Protein concentration was determined with a BCA kit (Pierce, Rockford, IL, USA). Equal amounts of total protein were subjected to SDS-polyacrylamide gel electrophoresis, and blotted to nitrocellulose membranes. The blots were blocked and incubated with anti-survivin antibody (Santa cruz clone D-8, sc-17779) as described before [16]. Blots were subsequently incubated with HRP-conjugated secondary antibody and detection was done using enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

Flow cytometry

Cell cycle determination was done by flow cytometry analysis using propidium iodide (PI), according to Vindeløv [17]. Cell cycle phases were calculated with Synchron Wizard. Immunocytochemical stainings for Cyclin B1, Cyclin A, and histone H3Pser10 were performed as described earlier [18]. One million HT29 cells were fixed with 1 % paraformaldehyde in PBS for 5 minutes on ice, spun down at 500 g and permeabilized by incubation in ice cold methanol for 10 minutes, spun down again and washed twice with 1%BSA/0.05%Tween/PBS (BSA/PBST). The cell pellet was incubated with primary antibody in BSA/PBST for 1 hour at RT: [Cyclin B1, Dako V152 (1:40); Cyclin A, BD Biosciences, Belgium (1:6); and histone H3Pser10, Sigma (1:100)]. Cells were washed twice with PBST, incubated with 100 µl goat-anti-mouse-FITC (1:100 in BSA/PBST, Dako), and

washed again. DNA staining was performed with 100 μM PI and 0.1 % RNAse in PBST for 30 minutes at RT. Cells were stored at 4°C until flow cytometric analysis on a FACScalibur (BD Biosciences) and pulse area vs. pulse width gating was performed to exclude doublets from the G2/M region. FITC fluorescence was collected in a log mode (FL1) and PI fluorescence in a lin mode (FL2) with 50,000 events stored for each sample. Apoptosis was also determined in pooled adherent and floating cell fractions by Annexin V/PI flow cytometry analysis according to the manufacturer (Sigma) with Annexin in FL1 and PI in FL2.

Statistical analyses

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). For the comparisons the ANOVA, Student's t-test with separate variance analyses were used.

Results

5-ASA inhibited the growth of CRC cells

We evaluated the effect of 5-ASA on the proliferation of the CRC cells HT29, Caco2 and HCT-116 *in vitro*. Cells were challenged with increasing concentrations of 5-ASA, within a therapeutically relevant range (0-50 mM) for 24, 48 and 72 hours. Seventy-two hour incubations with 5-ASA concentrations of 20 mM and higher significantly reduced HT29 cell proliferation (*Figure 1A*, P=0.0001). Concentrations above 35 mM even reduced cell proliferation levels below starting values after 24 hours, suggesting induction of cell death (*Figure 1B*). IC_{50} values, calculated from the MTS assays, decreased over time, with values of ~32, 30 and 27 mM for 24, 48 and 72 hour exposures, respectively, for HT29 cells. Comparable IC_{50} values were found in other CRC cell lines, Caco2 and HCT116 (*Table 1*). Thus, 5-ASA inhibited CRC cell proliferation in a concentration- and time-dependent manner. Interestingly, incubations with 5-ASA in enema formulation showed a similar IC_{50} in HT29 cells at 72 hours (32.17 mM), indicating minor/no influence of other preserving substances present in the enema solution.

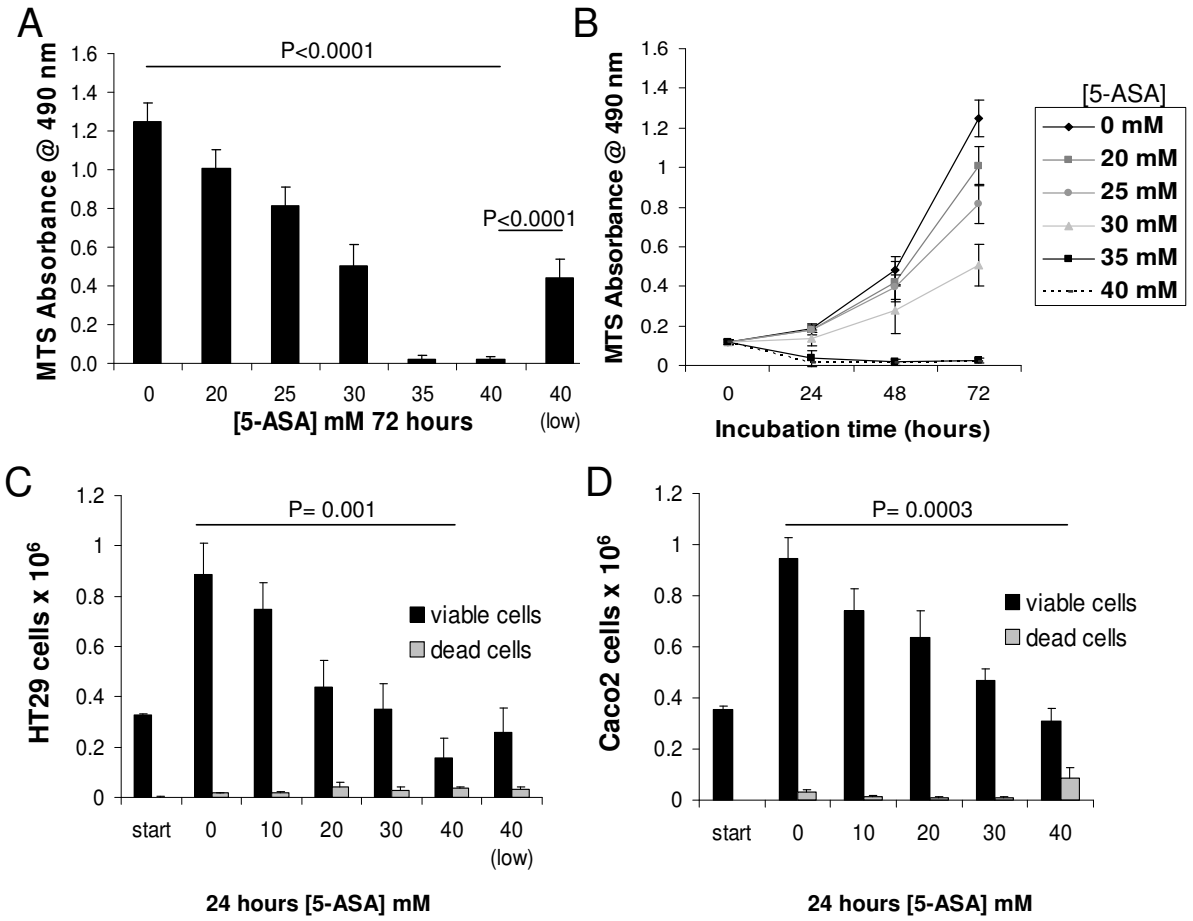

Figure 1: 5-ASA reduces CRC cell growth

MTS data of HT29 cells treated with 5-ASA (mM) for 72 hours (A). 24, 48 and 72 hour MTS data of HT29 cells treated with 5-ASA (B). MTS data were confirmed by cell counting of HT29 (C) and Caco2 cells(D) after 24 hours 5-ASA treatment. All data are expressed as mean + SEM of at least 3 independent experiments. Statistical significance was calculated using ANOVA and Student's t-tests.

Accidentally we discovered that the effects of 5-ASA were not only concentration but also dosage dependent. In 96-wells cultures the 100 µl dosage of 40 mM 5-ASA on 2 x 10^3 HT29 cells reduced the MTS absorbance to background levels, indicating almost no viable cells to exist, while 25 µl of 40 mM 5-ASA on 2 x 10^3 HT29 cells does not fully inhibit cell proliferation [*Figure 1A*, 40 (low)]. Because 100 µl of 20 mM 5-ASA does not reduce MTS absorbance as much as 25 µl of 40 mM 5-ASA it is not only the dosage of 5-ASA that is important for the effect but also the 5-ASA concentration. Viable cell counting of HT29 and Caco2 cells incubated with 5-ASA for 24 hours agreed with the MTS assay, growth inhibition by 20 and 30 mM 5-ASA and a reduction of viable cell numbers below starting levels by 40 mM 5-ASA (*Figure 1C/D*). It also confirmed a difference in cell growth inhibition of the two dosages of 40 mM 5-ASA.

Table 1: IC_{50} values for 5-ASA with respect to proliferation in CRC cells.

Cell line	24 hours	48 hours	72 hours
HT29	31.65 ± 0.37	29.67 ± 0.51	26.71 ± 0.40
Caco2	26.48 ± 1.84	18.42 ± 1.94	17.05 ± 0.84
HCT116	31.42 ± 0.81	24.31 ± 1.00	21.68 ± 0.67

IC_{50} values for 5-ASA (mM) in HT29, Caco2 and HCT116 colorectal cancer cells for 24, 48 and 72 hours.

5-ASA induced a cell cycle arrest

HT29 cells going through mitosis round up and loose attachment to the tissue culture plate. 5-ASA treatment increases the percentage of cells with this morphology (*Figure 2A*). To determine the phase of the cell cycle at which 5-ASA exerts its growth inhibitory effect, exponentially growing HT29 cells were treated with different concentrations of 5-ASA for 24 hours and flow cytometry analysis showed that 5-ASA increases the percentage of HT29 cells in the G2/M phase a 2 fold, from 13 to 27 % (*Figure 2B*, P=0.005). This resulted in less cell growth after longer incubation times indicating that cells were arrested in the G2/M phase, with less growth as a consequence. Cytospins were prepared and the nuclei of HT29 cells were stained with haematoxylin, to identify dividing cells by morphology. Mitotic cells were counted by light microscopy, with binuclear cells counted separately. Twenty-four hour incubation with a concentration of 20 and 30 mM of 5-ASA increased the percentage of cells in mitosis up to 2-fold compared to control cells (*Figure 2D*). The percentage of binuclear cells increased similarly indicating that 5-ASA not only effects mitosis (the division of the nucleus) but also cytokinesis (the splitting of the daughter cells).

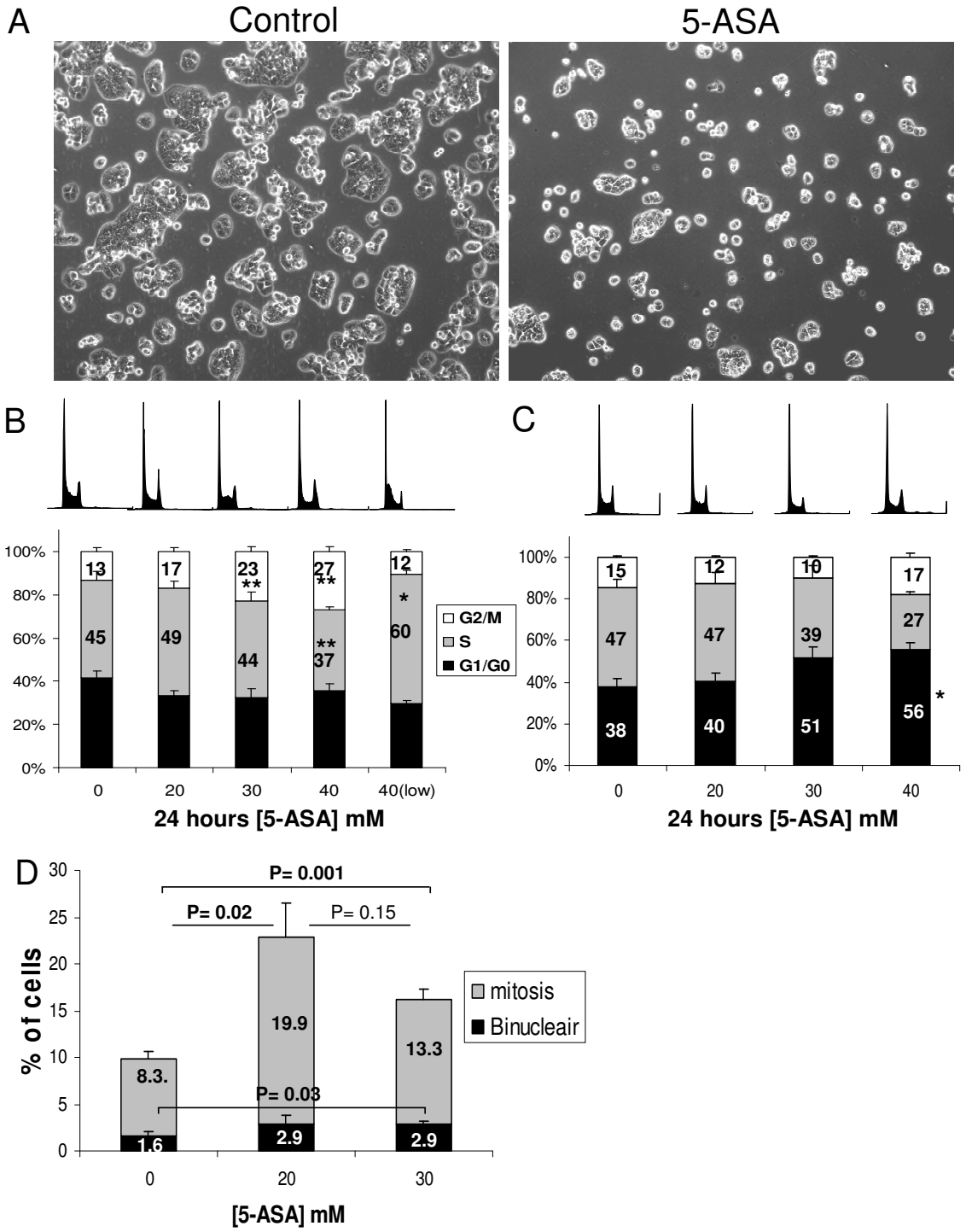

Figure 2: 5-ASA induces a cell cycle arrest in CRC cells.

*Phase-contrast image of control (left panel) and 24 hour 40 mM 5-ASA treated HT29 cells (A, Original magnification 100 x). Cell cycle analysis of HT29 cells (B) and Caco2 cells (C) after 24 hours 5-ASA treatment. Percentages mitotic and binucleair HT29 cells after 24 hour 5-ASA treatment (D). All data are expressed as mean + SEM of at least 3 independent experiments. Statistical significance was calculated using ANOVA and Student's t-tests. * P<0.05, ** P<0.01.*

Interestingly, HT29 cells treated with the low dosage of 40 mM 5-ASA [40(low)] showed an S-phase arrest after 24 hours (*Figure 2B*, P=0.02), rather than a G2/M arrest, again indicating different 5-ASA effects at different dosages.

In Caco2 cells there was a different effect, the percentage of S-phase cells decreased from 47 until 27 % (*Figure 2C*, P=0.01) and a simultaneous increase in G0/G1-phase cells from 38 % up to 56 % (P=0.02).

5-ASA induces cell death

The exposure of CRC cells to 5-ASA at concentrations ≥35 mM lead to a reduction of the cell population proliferation levels below the starting value in the MTS assays. This suggests that the induction of cell death played a major role in the mechanism of 5-ASA to reduce cell growth. 5-ASA induced apoptosis, as determined by M30 immunohistochemistry, in HT29 cells at concentrations of ≥30 mM (*Figure 3A*). In Caco2 cells there was a similar induction of apoptosis, although this was not statistically significant (*Figure 3A*).

The secretion of M30 antigen into the medium, also indicative of apoptotic cell death, was increased by 40 mM 5-ASA treatment in both cell lines (*Figure 3B*). The release of unprotealized CK18 represents the leakage of intact structural proteins, a hallmark of cell necrosis. The release of total CK18 (either the M30 antigen or intact CK18) was also increased by 5-ASA treatment. The M30/CK18 ratio represents the balance between apoptosis and necrosis and decreased due to 5-ASA treatment indicating that there was an increase in necrosis vs apoptosis. This was confirmed by AnnexinV/PI flow cytometry. 5-ASA increased the percentage of Annexin V+/PI- (early apoptotic) HT29 cells from 4% to 14% by 24 hours 40 mM 5-ASA treatment (*Figure 3C*), indicating the induction of apoptosis. The percentage of AnnexinV+/PI+ (late apoptotic/necrotic) cells increased from 6% to 28% and suggest that 5-ASA also induced necrosis.

In Caco2 cells there also was an increase in early apoptotic cells (6% to 10%) and late apoptotic/necrotic cells (18% to 54%) by 40 mM 5-ASA treatment (*Figure 3C*). The larger percentage of dead cells in Caco2 compared to HT29 was also seen in the cell counting experiments (*Figure 1D*).

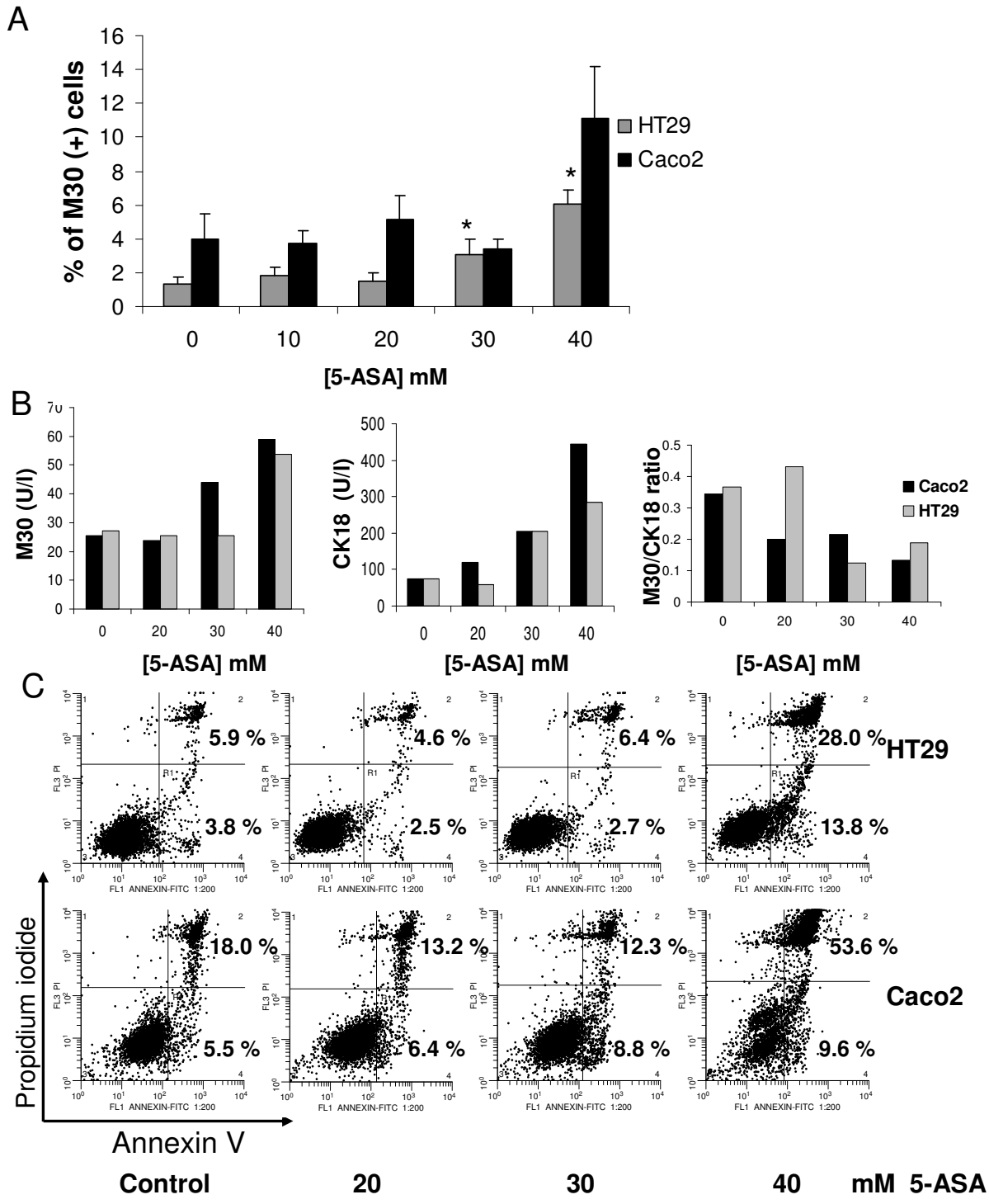

Figure 3: 5-ASA induces cell death.

*The percentage of apoptotic cells as determined by M30 immunohistiochemistry (A) All data are expressed as mean + SEM of at least 3 independent experiments. M30 antigen, total CK18 and the M30/CK18 ratio in the medium of HT29 and Caco2 cells treatd with 5-ASA for 24 hours (B). AnnexinV/PI flow cytometry of HT29 and Caco2 cells treated with 5-ASA for 24 hours (C).*P<0.05.*

Repeated 5-ASA treatment induces apoptosis and mitotic catastrophe

To determine if 5-ASA permanently arrested HT29 cells in the cell cycle HT29 cells were treated with 5-ASA for 24 hours, washed with PBS twice and re-seeded in equal cellular amounts in standard culture medium, allowed to adhere and grown for an additional 72 hours. HT29 cells treated with 20-30 mM 5-ASA were able to proliferate as normal cells (*Figure 4A*), indicating that the cell cycle arrest at these concentrations was reversible.

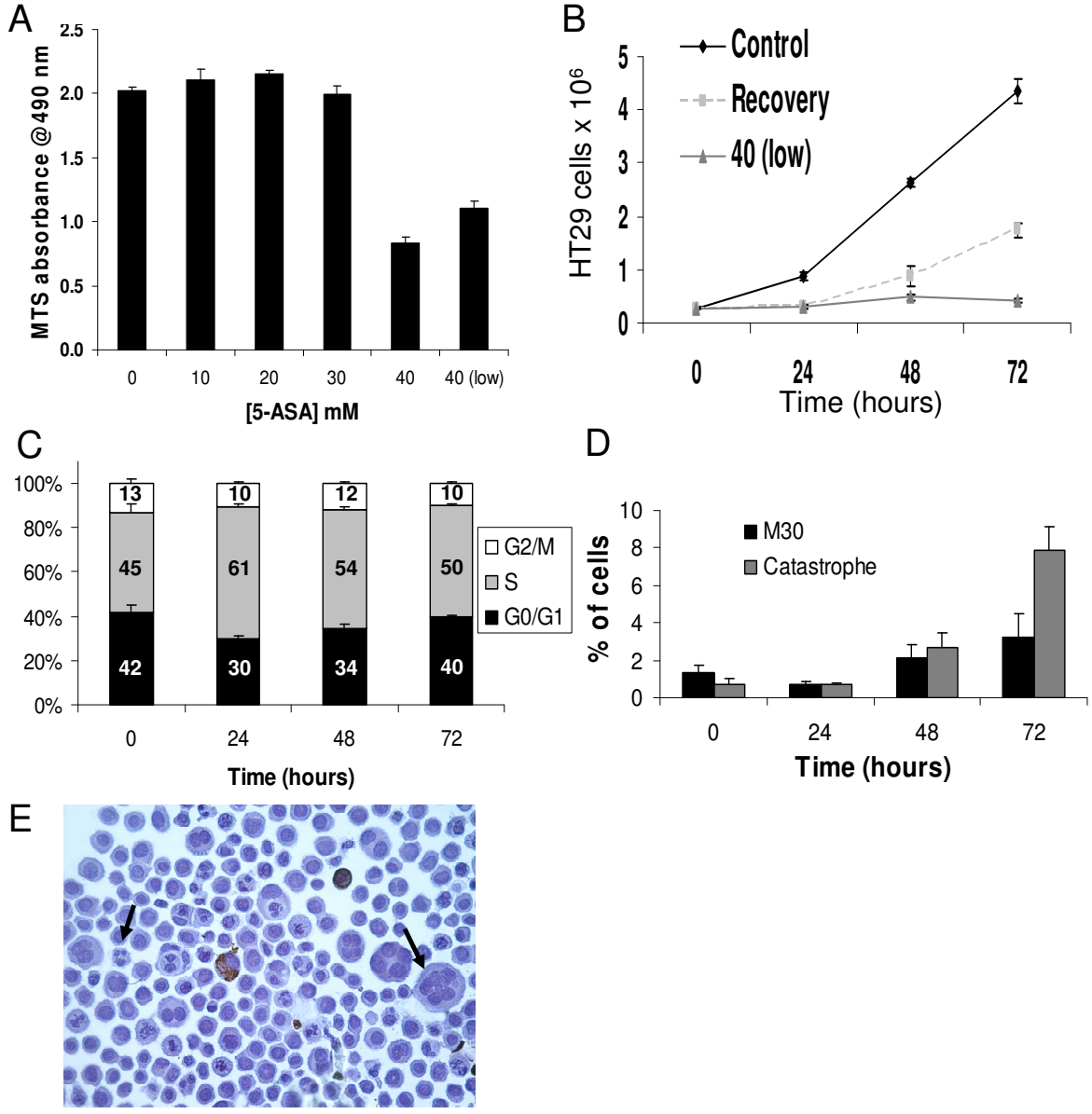

Figure 4: The 5-ASA induced cell cycle arrest in HT29 cells is reversible and irreversible.

MTS data of HT29 cell recovery after 24 hour 5-ASA treatment (A). HT29 cell numbers when the 5-ASA treatment is prolonged or stopped (B). HT29 cells are released from the cell cycle arrest upon prolonged treatment with 40 (low) 5-ASA treatment in time (C), with a simultaneous increase in apoptotic and mitotic catastrophic cells (D). All data are expressed as mean + SEM of at least 3 independent experiments. M30 positive (apoptotic, brown) and mitotic catastrophic cells (indicated by arrows) are shown (E, original magnifaction 100 x). Full-colour image on page 183.

HT29 cells treated with 40 mM 5-ASA, either the low or high dosage, were not able to proliferate similar to non-treated cells when re-plated in standard growth medium, indicating that the cell cycle arrest at 40 mM 5-ASA was (at least partly) irreversible (*Figure 4A*). Repeating the low-dosage 40 mM 5-ASA treatment in HT29 cells kept cells in a non-proliferative state, as there is no net cell growth (*Figure 4B*). Flow cytometry data showed that the S-phase arrest at 24 hours faded away after longer incubations, indicating that these arrested cells re-entered the cell cycle or died (*Figure 4C*). The repeated daily 5-ASA treatment caused a simultaneous increase in apoptotic cells, and also led to the appearance of giant cells containing uncondensed chromosomes, resembling a different form of cell death, i.e., mitotic catastrophe (*Figure 4D/E*).

5-ASA does not abrogate the G2 checkpoint

Mitotic catastrophe has been reported to be a consequence of prolonged cell cycle arrest, and can for instance occur when the G2 cell cycle checkpoint is inhibited and cells enter the M-phase while the S-phase is not completed properly (premature mitosis) [19]. Therefore, we hypothesized that 5-ASA abrogated the G2 checkpoint and caused premature mitosis in HT29 cells. This would explain why we find both an S-phase and M-phase arrest and could also explain mitotic catastrophe features. Flow cytometry analysis by a specific H3Pser10 antibody, correlating with mitosis [20], showed there was no chromosomal condensation before the chromosomes were completely duplicated, i.e., no H3Pser10 expression during the S-phase (*Figure 5*, indicated by arrow in the middel upper panel), which would be an indication of premature mitosis. An increase of 4N/H3Pser10 positive (mitotic) cells was seen indicating a mitotic arrest besides the S-phase arrest. Prolonged 5-ASA treatment resulted in a large percentage of 4N/H3Pser10 negative cells (*Figure 5*, upper right panel). Flow cytometric analysis of cyclin A and B1, first showed an increase in 4N/cyclin A negative cells (*Figure 5*, middle panel), indicative of mitotic cells [21]. Prolonged 5-ASA treatment increased 4N/Cyclin B1 negative cells, indicative of G2 or telophasic cells [22]. Sorting these 4N/cyclin B1 negative cells revealed that those were mainly catastrophic cells (*Figure 5*, insert).

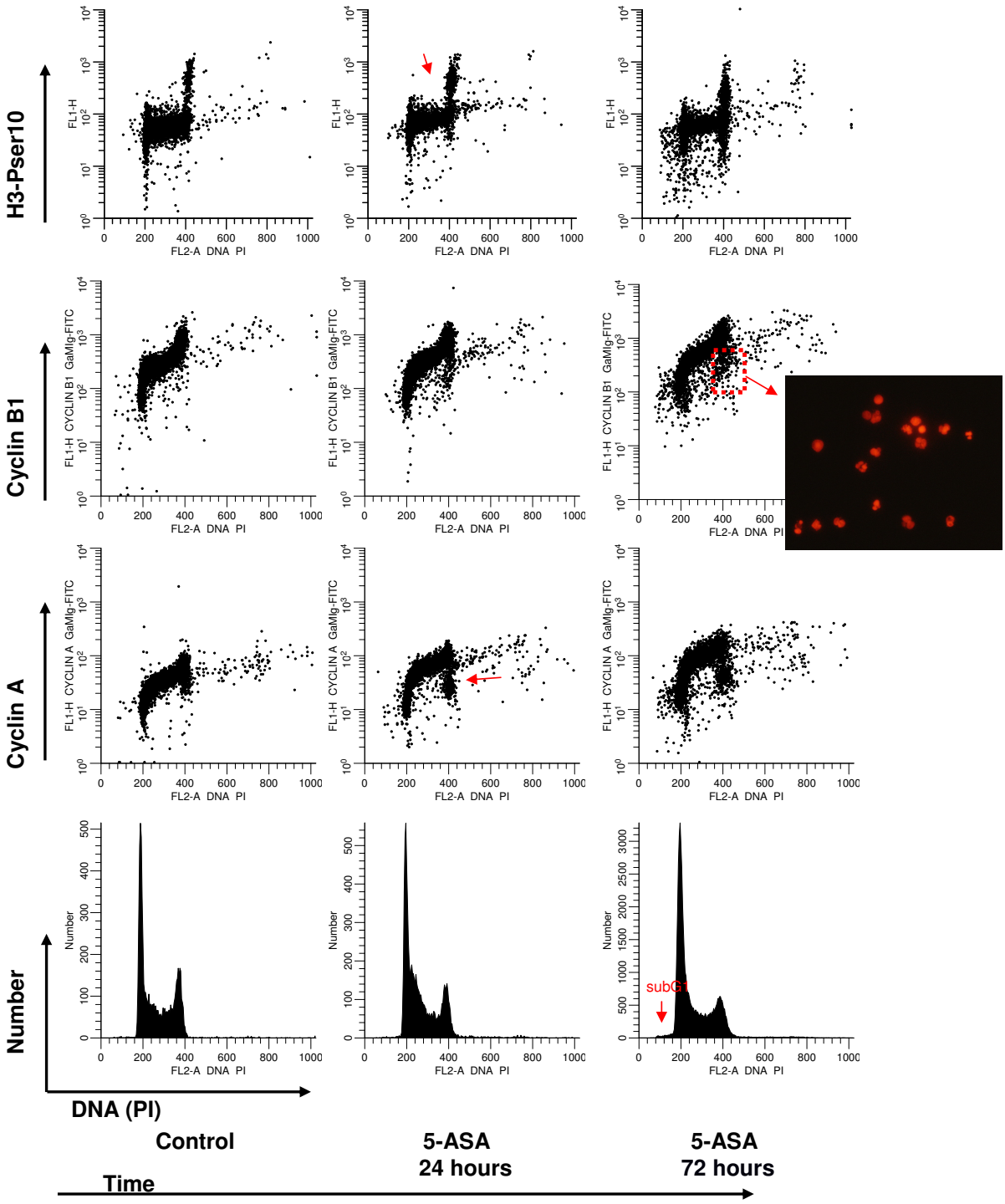

Figure 5: 5-ASA does not induce premature mitosis.

Cyclin A, Cyclin B1 and phospho-Histone H3 flow cytometry data for HT29 cells treated with 40 (low) mM 5-ASA for 24 and 72 hours. Inset showing sorted 4N Cyclin B1- cells, with mitotic catastrophe features. Original magnification 400 x. Full-colour image on page 181.

5-ASA causes mitotic spindle abnormalities

Microtubules are important cellular structures responsible for a proper segregation of chromosomes during mitosis. The organization of the mitotic spindle was found to be

abnormal in HT29 cells treated with low dosage 40 mM 5-ASA for 24 hours. Multipolar spindle structures (*Figure 6A*), not able to properly divide the chromosomes over two daughter cells, were found in 40 mM 5-ASA treated HT29 cells in contrast to control cells or cells treated with 20 mM 5-ASA, leading to a reversible arrest.

5-ASA does not affect the expression of survivin

Survivin, a member of the inhibitor of apoptosis (IAP) family, is an essential protein in cell division. Because survivin knock-down leads to delayed mitosis, misalignment of chromosomes, mitotic catastrophe, and apoptosis [23], very similar to the effects of 5-ASA described here, we investigated the effect of 5-ASA on survivin expression.

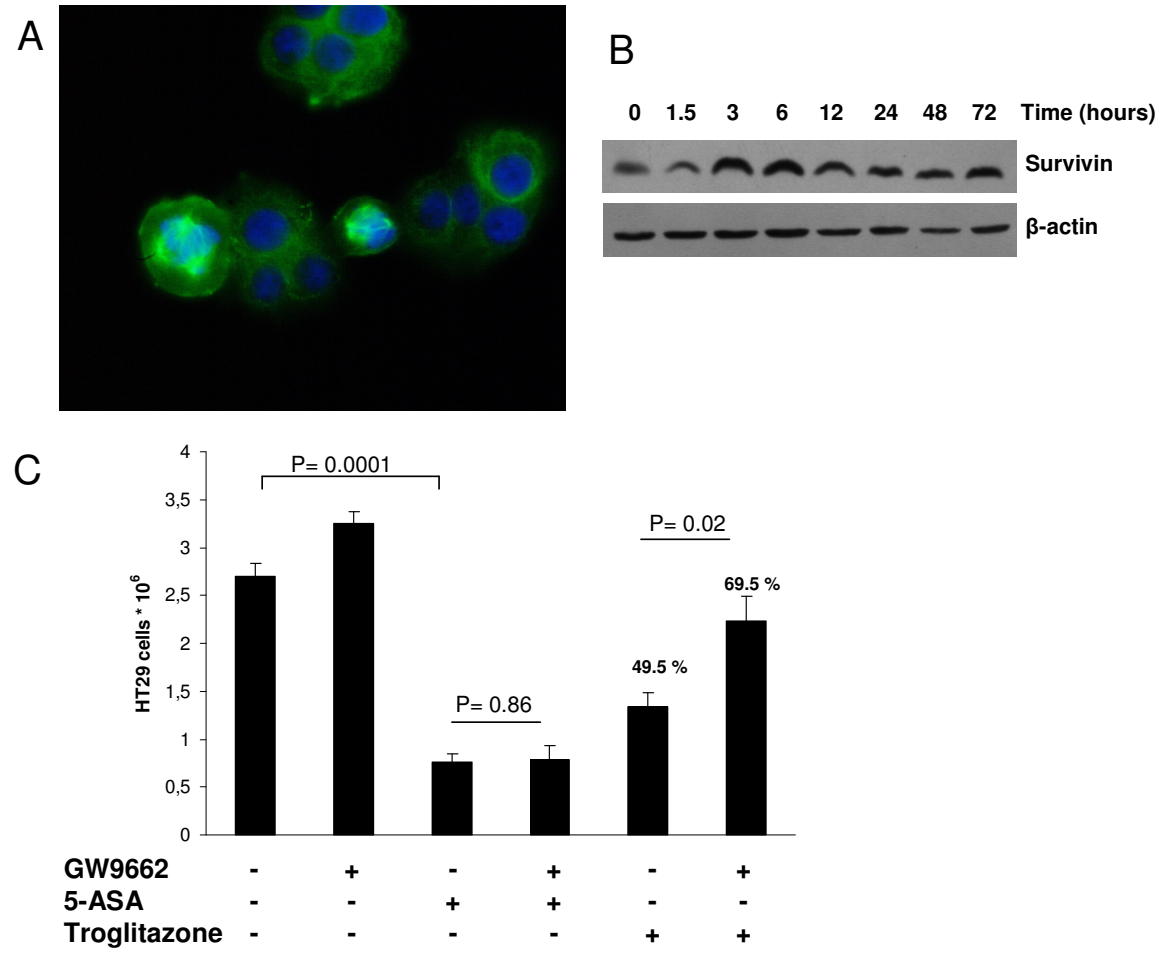

Figure 6: 5-ASA effects on the mitotic spindle, survivin and PPARγ.

5-ASA induces spindle abnormalities as shown by a merged image of 40 mM 5-ASA treated HT29 cells stained for α-tubulin (green) and DNA (blue). Original magnification 1000 x. Full-colour image on page 183 (A). Immunoblot analysis of survivin expression in HT29 cells treated with 40 mM 5-ASA over time (B). HT29 cell countings show that the anti-proliferative effects of 5-ASA are PPARγ independent. Mean + SEM of 3 independent experiments (C).

Surprisingly, 5-ASA treatment did not decrease but increased survivin protein levels in HT29 cell shortly after starting the 5-ASA treatment and dropped to basal expression levels in the subsequent time-period (*Figure 6B*). This may reflect the increase of the percentage of cells in cell division, expressing more survivin, at these early time-points.

5-ASA anti-proliferative effects are PPARγ independent

Given that 5-ASA is a peroxisome proliferation activated receptor (PPAR)γ agonist [24], and other PPARγ agonists have been reported to repress cellular proliferation [25, 26], it was of interest to know whether the 5-ASA anti-proliferative effects were PPARγ dependent. GW9662, a cell permeable high affinity antagonist of PPARγ [27], was not able to rescue the effect of 5-ASA on HT29 cell numbers, while it could efficiently block the effect of troglitazone (*Figure 6C*)**,** another well known PPARγ agonist [28].

Discussion

In the present study we found that 5-ASA is able to reduce the cell growth of various CRC cell lines, including p53 wildtype HCT116 cells, and p53 mutated HT29 and Caco2 cells. 5-ASA reduced cell growth in these CRC cells, in a concentration but also dosage dependent manner, by inducing various forms of cell cycle arrest and various forms of cell death. HT29 cells treated with a low dosage of 40 mM 5-ASA for 24 hours were arrested in the S-phase. When this 5-ASA treatment was prolonged, with daily refreshment of 5-ASA, some HT29 cells became apoptotic, others developed into giant cells containing multiple micronuclei and giant multilobular nuclei. The presence of those abnormal cells accounts for the induction of "mitotic catstrophe", a form of cell death that resulted from aberrant cell division. Aberrant cell divisions were indeed found at earlier time-points of this 5-ASA treatment, represented by multipolar mitotic spindle abnormalities. Mitotic catastrophe is potentiated by a number of chemical inhibitors of the G2 checkpoint, such as caffeine or okadiac acid, a protein phosphatase 2A (PP2A) inhibitor. Interestingly, 5-ASA was found to inhibit canonical *Wnt* signalling in CRC cells by PP2A inhibition [29]. However, our analysis of cyclin A and B1 and histone H3 phosphorylation did not provide any evidence that 5-ASA inhibited the G2 checkpoint, as 5-ASA did not cause premature mitosis in HT29 cells. It furthermore showed that besides an S-phase arrest 5-ASA also caused complications in the subsequent M-phase.

When a 4 times higher dosage of 40 mM 5-ASA was used to treat HT29 cells, there mainly was an induction of apoptotic and necrotic death in 24 hours. This was accompanied

by an increase in the percentage of cells in the G2/M-phase, in contrast to the S-phase seen with the lower dosage, suggesting that mainly the cells that entered the S-phase encountered problems due to the 5-ASA treatment and have died. The consequences of the prolonged (72 hours) low dosage 40 mM treatment, i.e., cell death, seemed to be forced into the shorter time period when a higher dosage was used.

In contrast to a 5-ASA induced increase in the S-phase or G2/M-phase cells in HT29, 5-ASA increased the percentage of G0/G1-phase cells in Caco2, in a concentration dependent manner. This G0/G1-arrest was accompanied by a massive induction of cell death by 5-ASA, much more pronounced than in HT29 cells, as judged by viable cell countings, M30 immunohistochemistry and AnnexinV/PI flow cytometry, which explains the lower IC_{50} values for 5-ASA in Caco2 cells. Cell cycle arrest, particularly in the G0/G1-phase of the cell cycle, is known to be associated with the induction of cell death. Because Caco2 express catalytically active cyclooxgenase(COX)-2, in contrast to HT29 and HCT116 [30], the more pronounced effect of 5-ASA on Caco2 cells might be COX-2 related. However, similar anti-proliferative actions of 5-ASA in HT29 and HCT116 cells indicate that the effects are mainly COX-2 independent, which is in line with a recent report [31].

M30 immunohistochemistry and M30 antigen secretion indicated that 5-ASA induced apoptosis. The M30/CK18 ratio decreased due to 5-ASA treatment indicating that there was an increase in necrosis, besides the increase in apoptosis. The induction of apoptosis is normally characterized by DNA fragmentation resulting in a characteristic DNA ladder pattern and a so called sub-G1 fraction in the flow cytometry histograms, which is used by many researchers to quantify apoptosis. 5-ASA treatment does not result in high numbers of cells with this feature, as reported by others [32], and therefore does not seem to induce this so called classical apoptosis. Mitotic catastrophe lacks this typical apoptotic DNA fragmentation, and might be the most important pathway in 5-ASA induced death.

In the present study, we showed that not only the concentration but also the dosage of 5-ASA is important for the cellular response, which could also be true for other NSAIDs. Because 5-ASA is acetylated by cells into the metabolically inactive Ac-5-ASA [33, 34] and oxidizes when dissolved in culture medium [35], the concentration of 5-ASA is higher for a longer time when a higher dosage is used. Because researchers usually do not indicate the dosage used in their experiments, it is difficult to compare results from different studies. Several kind of cell cycle arrests induced by 5-ASA have been described in the literature [32, 36-38], which might be related to dosages used. More importantly, we have to keep in mind that we try to mimic *in vivo* situations in *in vitro* experiments by using therapeutically relevant

concentrations of 5-ASA (and other anti-cancer medication). The question then arises whether the dosage is also therapeutically relevant. Example, in our MTS assays we treated 2 x 10^3 HT29 cells with a (low) dosage of 25 µl 40 mM 5-ASA, which is 7.7 x 10^{-8} gram 5-ASA per cell. In a patient on 4 gram 5-ASA treatment this dosage would only be to treat 5.2 x 10^7 cells, when we consider an equal dose as in our MTS assay, and shows that the dosage is not therapeutically relevant. Of course oxidation and acetylation circumstances are different in the intestine *in vivo*, and two reports have indeed shown that 5-ASA treatment reduces proliferation and induces apoptosis *in vivo* [39, 40].

Given that 5-ASA is a ligand for PPARγ, that is able to activate downstream signaling, and PPARγ agonists have been reported to repress cellular proliferation, it was of interest to investigate whether the 5-ASA anti-proliferative effects were PPARγ dependent. GW9662 was not able to block the anti-proliferative effects of 5-ASA while it was able to block the anti-proliferative effect of troglitazone, indicating the anti-proliferative effects of 5-ASA are PPARγ independent. A recent publication by Schwab *et al.* [37] showed that 5-ASA arrested Caco2, HT29 and HCT116 cells in the G0/G1-phase of the cell cycle and induced apoptosis, in a manner that partly depended on PPARγ, in contrast to our results.

In conclusion, we showed that 5-ASA interferes in the cell cycle of CRC cells and induces cell death, in the form of apoptosis, mitotic catastrophe and necrosis, which is a desirable goal in anti-cancer treatment.

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