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

Koelink, P.J.

Chapter 8

Radioprotection of colorectal cancer cells by 5-aminosalicylic acid

Pim J. Koelink, Johanna A.M. van der Zon, Daniel W. Hommes, Hein W. Verspaget

Department of Gastroenterology-Hepatology
Leiden University Medical Centre
Leiden, The Netherlands

Abstract

5-Aminosalicylic acid (5-ASA, mesalazine) is a non-steroid-anti-inflammatory drug (NSAID) that is widely used in the treatment of inflammatory bowel diseases. There is compelling evidence showing that 5-ASA is also an interesting candidate drug in a treatment strategy against colorectal cancer (CRC), due to its diverse anti-cancer effects. Patients with CRC are treated with radiation therapy (RT) to decrease disease recurrence and improve respectability and survival. We aimed to investigate if 5-ASA treatment has effect on RT in CRC cells. We therefore determined the overall and clonogenic survival of HT29 and HCT116 cells *in vitro*, after a combinational treatment of 5-ASA and RT or subsequent RT after 5-ASA treatment. RT in the presence of 5-ASA was less effective than RT alone, in both HT29 and HCT116 cells, indicating a radio-protective effect by 5-ASA. This radio-protective effect depended on the 5-ASA concentration used with a dose-mutation factor of 1.09-1.19. Radiation therapy immediately after 5-ASA treatment was also less effective, indicating that not 5-ASA itself but changes in down stream targets play an important role in its radio-protective activity. These changes were not permanent because CRC cells are as sensitive to RT 24 hours after 5-ASA treatment had been stopped. We therefore conclude that 5-ASA reduces the sensitivity of CRC cells to RT by effects on intracellular down stream targets, in a reversible manner.

Introduction

Colorectal cancer (CRC) is a common malignancy in the Western world and is responsible for 500.000 deaths worldwide each year [1]. The development of local recurrence after surgery, especially in rectal cancer, is a major problem and is also difficult to treat [2]. To decrease the local recurrence rate and to improve survival radiotherapy (RT) is one of the major neo-adjuvant therapies used in rectal cancer. Pre-operative RT, followed by surgical resection, improves respectability as well as the risk of local recurrence and survival rates by 51% and 16%, respectively, when compared with surgery alone [3]. There is a wide variety in tumour response to RT, either alone or in combination with chemotherapy (radiochemotherapy). RT induces apoptosis and other types of cell death, like necrosis [4]. In recent years a lot of research is done to increase the radiation effects on cancer cells by studying compounds that enhance the effects of radiation, i.e., radiosensitizers, or limit the (side-)effects on normal cells [5].

There is ample evidence that 5-ASA, a non-steroid-anti-inflammatory-drug (NSAID) widely used in the treatment of inflammatory bowel diseases (IBD), possesses anti-cancer effects both *in vitro* and *in vivo.* 5-ASA inhibits proliferation and induces apoptosis of CRC cells and it is able to inhibit various pathways that are activated in cancer development/progression, including the *Wnt* and the NFκB pathway [6-15]. Furthermore, 5-ASA is also able to activate the anti-inflammatory PPARγ pathway, which is believed to be a tumour suppressor pathway [16, 17]. Because of an excellent safety and side-effects profile [18], 5-ASA is an interesting therapeutic compound for pre-operative treatment, since local treatment by enema enables a high luminal concentration of the drug [19], and because it has been shown to induce apoptosis in CRC cells *in vivo* [8]. 5-ASA also acts as a oxygen radical scavenger [20, 21], decreases the spontaneous mutation rate of cells by improving replication fidelity [22], and protects cells from DNA damage by 9-aminoacridine, an intercalating mutagen (C. Campregher, Falk symposium 158: Intestinal inflammation and colorectal cancer). In combination, with this activity profile it might well be possible that 5-ASA reduces RT effects in CRC. In addition, 5-ASA treatment was found to increase the survival of mice subjected to lethal radiation doses, whereas the survival of irradiated animals with transplanted Ehrlich ascites carcinoma (EAC) cells was not affected by 5-ASA treatment [23], suggesting that 5-ASA protects normal mouse cells, but does not protect carcinoma cells.

In the present study we investigated the effect of 5-ASA on the sensitivity of CRC cells to RT by treating two CRC cell lines, having different intrinsic radio sensitivities, with

5-ASA and RT *in vitro* and found that 5-ASA is able to protect CRC cells to RT in a reversible manner.

Material&Methods

Cell cultures and reagents

HCT116 (hMLH1 mutant) and HT29 (mutant $p53^{R273H}$) CRC cells were obtained from the ATTC and cultured in Dulbeco's Modified Eagle Medium DMEM/F12 (1:1) + GlutaMAX medium (Invitrogen, Breda, the Netherlands) supplemented with 10 % heat inactivated Fetal Calf Serum (FCS, Perbio Science, Belgium), 10 mM HEPES, 100 U/ml penicilin, 100 μg/ml streptomycin and 50 μg/ml gentamycin (all Invitrogen). 5-ASA (Dr. Falk Pharma, Freiburg, Germany) was dissolved in culture medium, pH adjusted (~7.4) and filter-sterilized.

Radiation therapy

HT29 and HCT116 cells were trypsinized, viable cells counted by trypan blue exclusion and diluted in plastic 15 ml tubes. The cells were irradiated in suspension with a range of doses (2-6 Gy) using ^{137}Cs γ-rays at a dose rate of ~8.8 Gy/min.

MTS Survival assays

To determine the proliferative capacity of cells we performed 96-well format proliferation assays. Cells were irradiated in a 5000 cells/ml suspension with 5 Gy of radiation and seeded at 1000/well (200 μl) in quadruplicate. Non-irradiated cells served as controls. The net cell growth of these cells was monitored after 5 days of culture by the AQ_{ueous} One Solution Cell Proliferation Assay, according to manufacturer's instructions (Promega, Madison, WI, USA). This assay assesses cell viability by measuring the metabolic activity of cells through conversion of MTS (soluble tetrazolium salt) by NADPH/NADH into a coloured formazan product. Briefly, 20 μl of the MTS reagent was added to 100 μl medium on cells. The absorbance was read at 490 nm in a 96-well plate reader (Molecular Devices, $Thermo_{max}$ microplate reader) after an incubation period of 2 hours.

Clonogenic survival assays

To demonstrate the effect of 5-ASA on the radiation sensitivity of CRC cells, HT29 and HCT116 cells were seeded in triplicate into six-well plates, immediately after RT, in a

range of 100-5000 cells per well, depending on the radiation dose the cells received. Cells were cultured for 11-12 days and medium was refreshed after 5-6 days, before cells were fixed and stained for 45 min with a solution containing 0.25 % methylene blue (Sigma Aldrich Chemicals, Germany) in 50 % ethanol. Colonies (>50 cells) were counted, normalized against non-irradiated control cells and the data were analyzed in a linear semi-log model with Graphpad Prism (version 5.0, Graphpad Prism Inc., La Jolla, CA, USA) software. The dose mutation factor (DMF) was calculated by dividing the RT dose necessary for a 95 % decrease in survival for 5-ASA treatment versus untreated cells.

Results

5-ASA protected CRC cells from RT

CRC patients usually receive RT doses of 5 Gy, approximately 4 to 5 times. HCT116 and HT29 cells were therefore also irradiated with 5 Gy of RT in the absence or presence of different concentrations of 5-ASA. HT29 and HCT116 were confirmed to have different intrinsic radiosensitivities, 5 Gy RT decreased the net cell growth of HT29 and HCT116 cells to ~30 % and ~10 % respectively (*Figure 1A*), with HT29 cells being less radiosensitive most likely due to the p53 mutation status.).

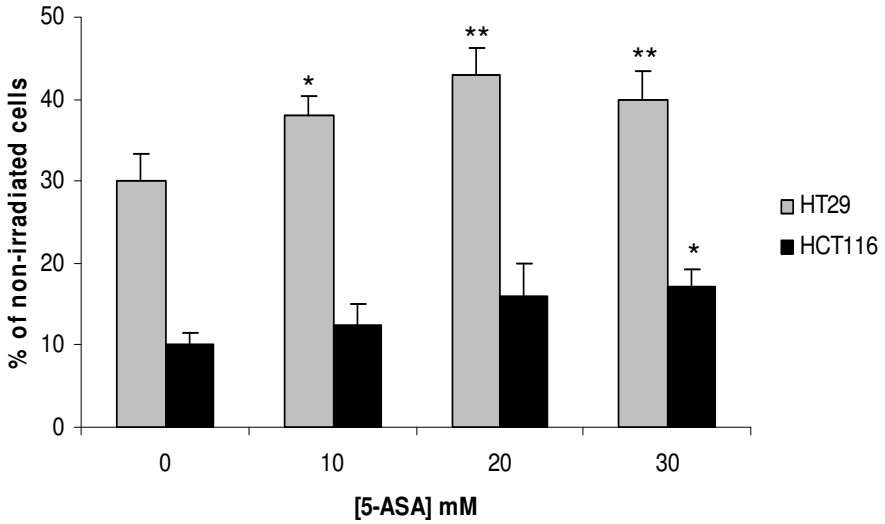

Figure 1: 5-ASA protected CRC cells from RT.

*5-ASA increased the percentage of surviving HT29 and HCT116 cells after 5 Gy of RT as determined in 3 independent MTS assays (mean+SEM). Groups were found to be statistically different as determined by ANOVA. Statistical significance was determined by the Student t-tests.*P≤0.05, **P≤0.01.*

5-ASA partially protected both CRC cell lines from a 5 Gy dose of RT and increased the growth and survival of HT29 cells from 30 to 38, 42 and 39% for 10, 20 and 30 mM 5-ASA, respectively (*Figure 1A*). The growth and survival of HCT116 was also increased from 10 to 12, 16 and 17% for 10, 20 and 30 mM 5-ASA, respectively.

Clonogenic survival assays, the golden standard to determine the cellular response upon RT, using 2, 4 and 6 Gy of radiation and non-irradiated controls showed similar results with respect to the radiosensitivity of the cell lines (*Figure 2*). In these assays 5-ASA was found to have a similar, more or less dose-dependent, protective effect on the RT related cell survival of HT29 and HCT116 cells. The DMF's at a survival of 0.05 were between 1.09 and 1.19 (*Table 1*), depending on 5-ASA concentration and cell line used.

Table 1: Dose mutation factors for different concentration of 5-ASA.

[5-ASA] mM	HT29		HCT116	
	RT dose (Gy) at 0.05 survival	Dose mutation factor	RT dose (Gy) at 0.05 survival	Dose mutation factor
0	4.74	-	3.32	-
10	5.28	1.11	3.68	1.11
20	5.66	1.19	3.63	1.09
30	5.25	1.11	3.94	1.19

Dose mutation factors were calculated from the from the RT dose (Gy) necessary to reduce clonogenic survival by 95 % (0.05) on HT29 and HCT116 cells.

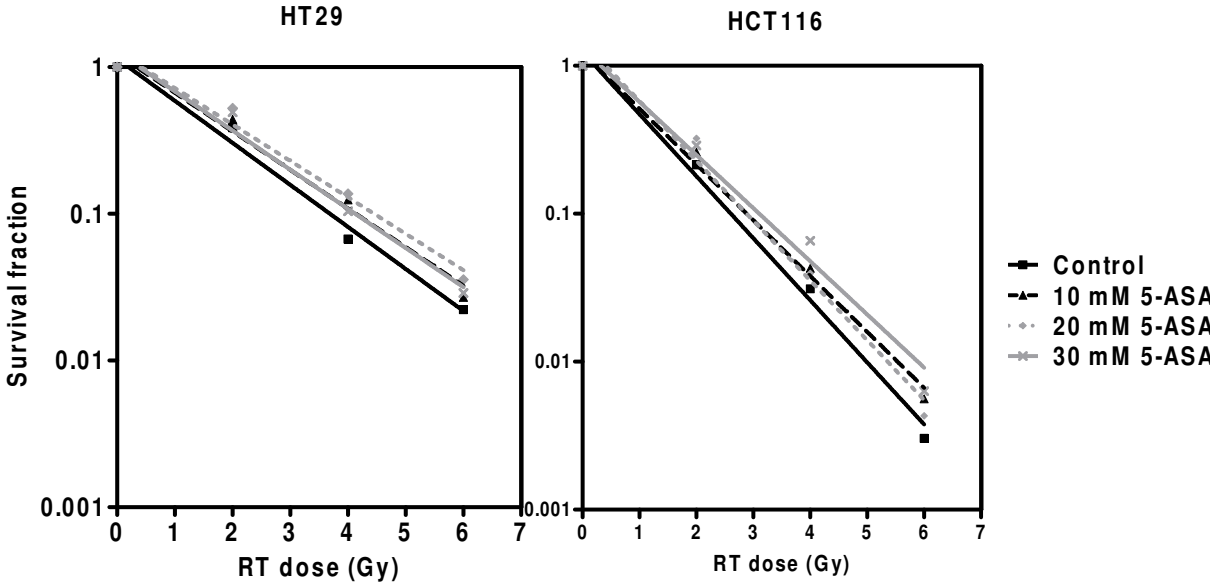

Figure 2: 5-ASA increased clonogenic survival of CRC cell lines.

The combined clonogenic survival of HT29 and HCT116 cells of 3 independent experiments is shown (mean, error bars are omitted because of clarity reasons).

5-ASA pre-treatment reduced sensitivity to immediate RT

We next investigated whether the presence of 5-ASA during the RT was responsible for the observed protection to RT, or that some downstream target proteins were responsible for this radio-protective effect. The cells were pre-treated with 30 mM 5-ASA for 24 hours, counted and extensively washed, exposed to RT treatment in the absence of 5-ASA, and plated in equal cell numbers. 5-ASA decreased cellular proliferation (*Figure 3A*), with both cell lines being about equally sensitive, as reported before [11]. Cells that were treated with 30 mM 5-ASA for 24 hours were able to proliferate as untreated cells after reseeding (*Figure 3B*), as reported before [11, 13].

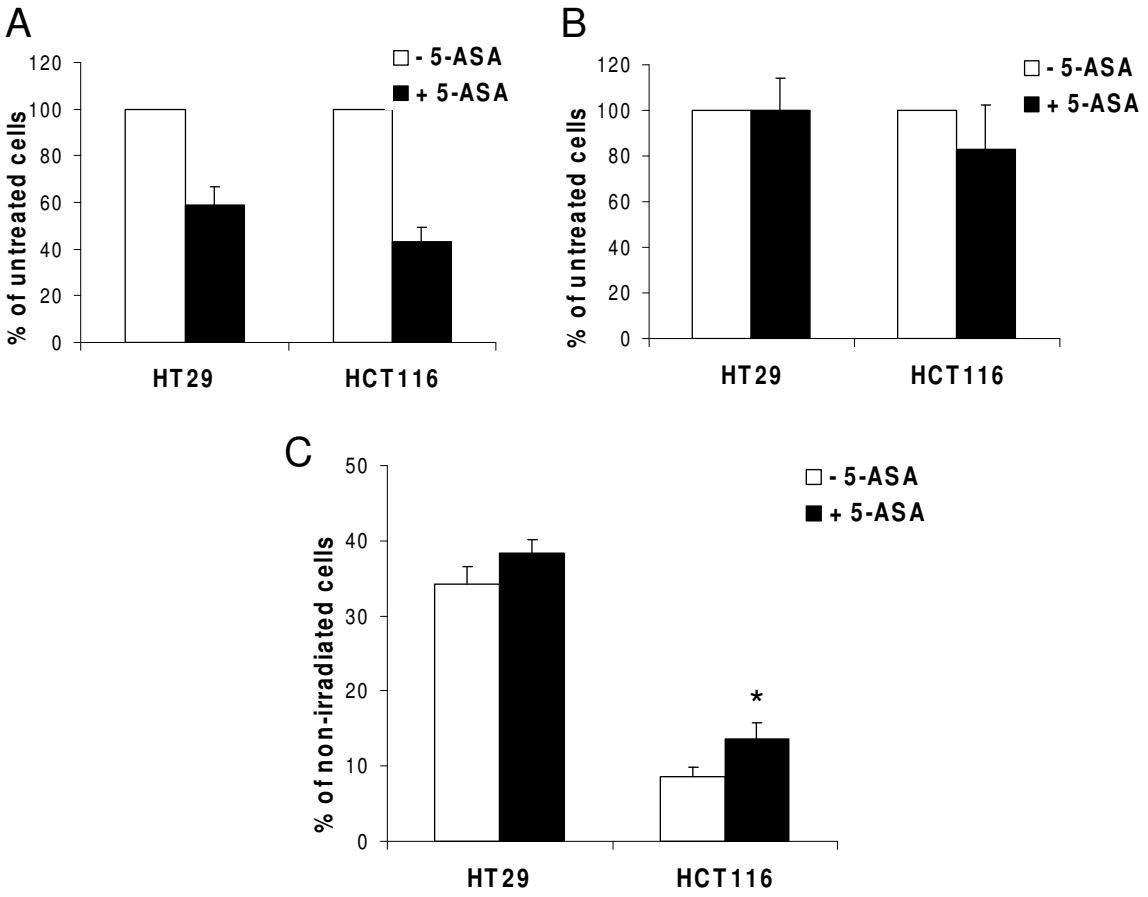

Figure 3: 5-ASA pre-treatment protected CRC cells from subsequent RT.

(A) 30 mM 5-ASA decreased the proliferation of HT29 and HCT116 cells. The cell were counted and expressed as the percentage of untreated cells of 3 independent experiments (mean+SEM). (B) HT29 and HCT116 cells were able to proliferate as untreated cells after 30 mM 5-ASA pre-treatment indicating reversible effects on proliferation. The percentage of non-treated cells is expressed as determined by 3 MTS assays (mean+SEM). (C) 5-ASA pre-treatment increased the percentage of surviving cells after 5 Gy of radiation as determined in 3 independent MTS assays (mean+SEM). Statistical significance was determined by the Student t-tests. $P \leq 0.05$.*

HT29 and HCT116 cells pretreated with 5-ASA were less responsive to RT (*Figure 3C, Figure 4*), with a DMF of 1.11 and 1.16 at survival 0.05 for HT29 and HCT116 cells, respectively, which is similar to the DMFs in the direct presence of 5-ASA.

5-ASA pre-treatment did not reduce sensitivity to RT

The fact that the anti-proliferative effects of 5-ASA at these concentrations are reversible did suggest that it is unlikely that 5-ASA caused permanent changes in the genetic/proteomic make up of the cells. Treating cells for 24 hours with 5-ASA and releasing the cells from treatment for at least 24 hours, by culturing them in normal medium, enabled us to investigate whether the radio-protective effects of 5-ASA were permanent or reversible. There was no difference in the sensitivity to radiation (*Figure 5A,B*), either on overall survival or clonogenecity, when cells were allowed to recover from the 5-ASA treatment for 24 hours.

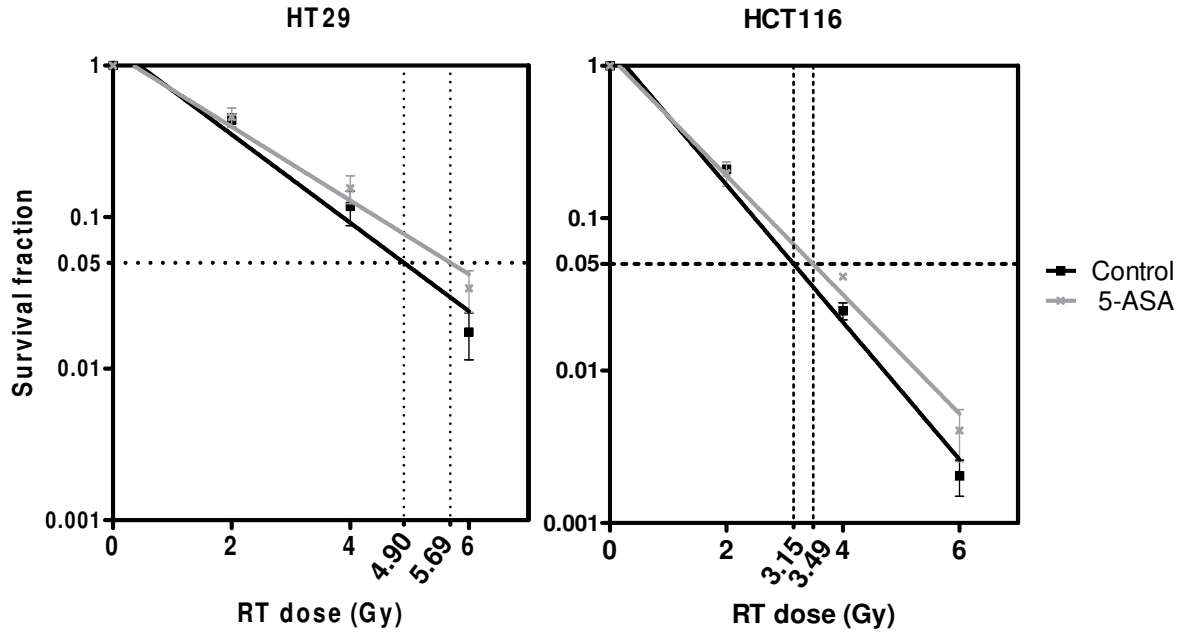

Figure 4: 5-ASA increased clonogenic survival of CRC cell lines.

Combined clonogenic survival of 3 independent experiments is shown (mean±SEM) and RT doses to reduce clonogenic survival to 5% of both 5-ASA treated and non-treated HT29 and HCT116 cells are indicated on the x-axis and used to calculate the DMF.

Discussion

The anti-cancer properties of 5-ASA combined with an excellent safety profile, make it an interesting candidate for the adjuvant treatment of CRC. 5-ASA reduces proliferation and induces cell death via mitotic catastrophe and apoptotic pathways [9, 11, 13, 15], very similar to the effects of chemotherapeutic agents used in the clinic nowadays. It is possible that 5-

ASA in combination with other therapies works even better. It has to be kept in mind; however, that 5-ASA treatment might also interfere in a negative way with therapeutic treatments that are already clinically applied to the patients. In the past several substances have been discovered and described that have the capability to sensitize cancer cells to RT, i.e., radiosensitizers. In this respect, we investigated the effect of 5-ASA treatment on RT of CRC cells *in vitro* by treating CRC cell lines, with different intrinsic radiosensitivities.

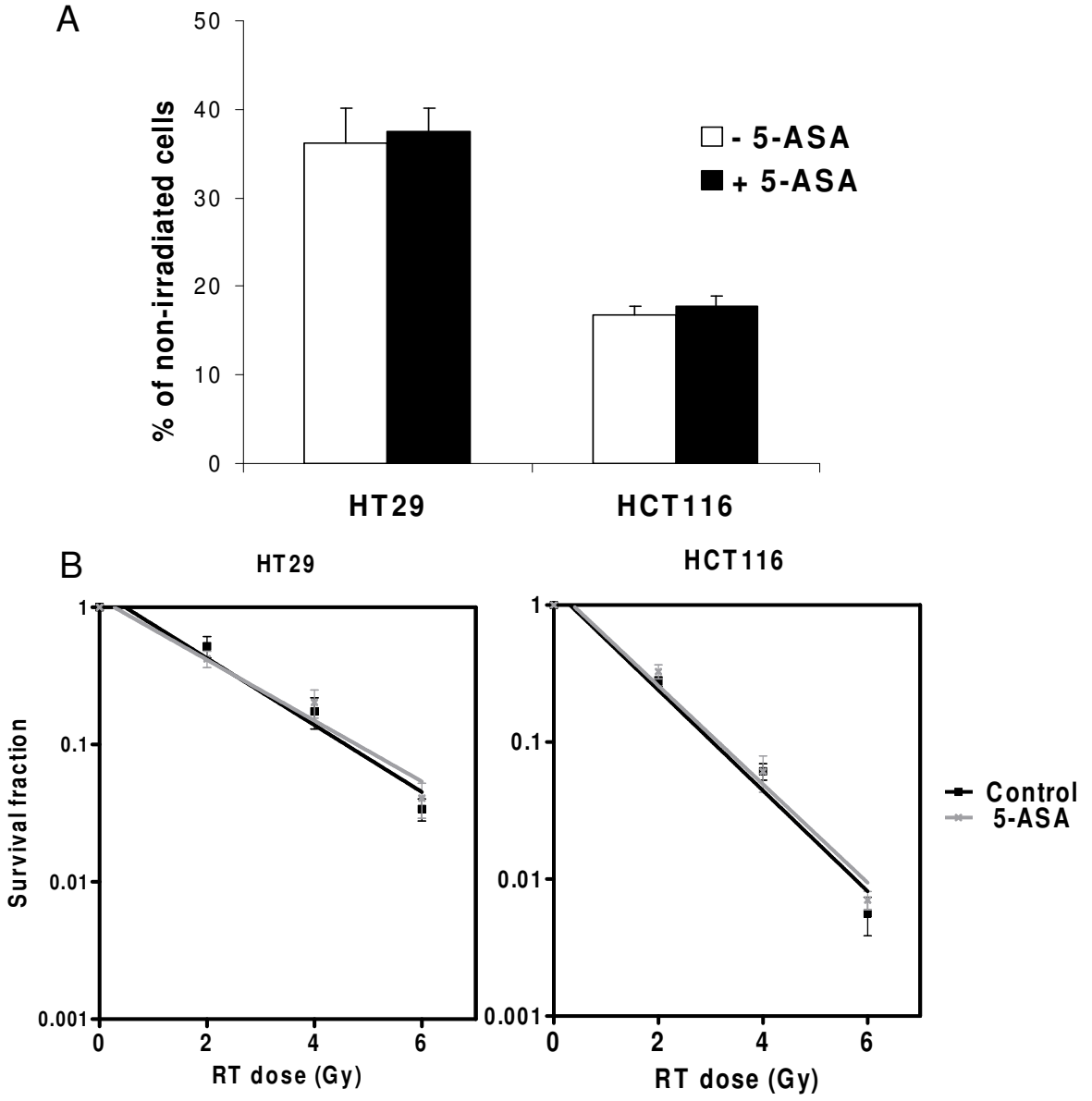

Figure 5: Reversibility radio-protective effect of 5-ASA.

(A) Allowing CRC cells to recover from 5-ASA treatment for 24 hours re-sensitizes them for RT. 5-ASA did not increase the percentage of surviving cells after 5 Gy of RT as determined in 3 independent MTS assays (mean+SEM). Statistical difference was determined by the Student t-test. (B) 5-ASA recovery did not increase clonogenic survival of CRC cell lines. Combined clonogenic survival of 3 independent experiments is shown (mean±SEM).

The results of the present study demonstrate that RT in the direct presence of 5-ASA is less effective than RT alone, indicating that simultaneous 5-ASA treatment provides a protective effect on CRC cells to RT. 5-ASA had a dose mutation factor of 1.09-1.19 at survival 0.05, depending on the cell line and 5-ASA concentration. This means that the standard 5 Gy dose under 5-ASA conditions must be increased by a factor 1.09-1.19 to 5.45-5.95 Gy to have the same survival decreasing effect on CRC cells. The DMF of 5-ASA is in the range of natural occurring radioprotectors, like plant and herbal compounds and is similar to the DMF of 1.08 reported for the protective effect of 5-ASA on the radiation of normal mouse cells [23]. 5-ASA treatment immediately followed by RT, so without the direct presence of 5-ASA during radiation, shows similar protection to radiation, indicating that not 5-ASA itself but downstream targets are most likely responsible for the radio-protective effect. The effect of 5-ASA is similar in HT29 and HCT116 cells suggesting that the effects are p53 independent, as HT29 cells in contrast to HCT116, lack wild type p53.
Allowing the cells to recover from the anti-proliferative and cell death inducing effects of 5-ASA first, did not offer protection to RT, indicating that the radio protective effect of 5-ASA is not permanent but reversible. This also suggests that 5-ASA exerts its anti-proliferative and cell death inducing efficacy on both RT resistant and RT sensitive cells. If 5-ASA treatment would have altered the ratio between RT resistant and RT sensitive cells this would have led to difference in sensitivity to subsequent RT, but this did not occur.

Spontaneous apoptosis rates in rectal cancer have been found to predict local recurrence *in vivo*, with low rate tumours developing more recurrence [24, 25]. The increased apoptotic rate due to RT, however, did not correlate with local recurrence rates [24]. Interestingly, 5-ASA induces apoptosis in CRC cells *in vivo* [8], but can also interfere with RT *in vivo*. In this report we showed that 5-ASA cannot be used in combination with RT, at least not simultaneously. Even when 5-ASA is used until just before RT this might desensitize CRC cells for RT treatment. However, our results demonstrate that if 5-ASA treatment is stopped in time, at least 24 hours before RT, the 5-ASA treatment is unlikely to interfere with it.

There is enough compelling evidence showing the anti-cancer efficacy of 5-ASA to start treating CRC patients with 5-ASA pre-operatively. We report here that 5-ASA treatment does not necessarily interfere with RT, an important existing CRC treatment. This provides an important experimental base for the treatment of CRC patients pre-operatively with 5-ASA immediately after diagnosis and for as long as possible, with a stop in treatment at least

a day before and certainly during RT. A controlled trail is indicated to assess the effect of 5-ASA, either in combination with or without current therapies, on the clinical outcome of the CRC patient: overall survival, disease-free survival, and disease recurrence.

5-ASA reversibly reduces CRC cell sensitivity to RT by effects on intracellular down-stream targets.

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