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The evaluation of the efficacy and potential genotoxic hazard of combined SAHA and 5-FU treatment in the chemoresistant colorectal cancer cell lines

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ABSTRACT

5-Fluorouracil (5-FU) is an essential chemotherapeutic drug for colorectal cancer (CRC) treatment. However, the frequent development of drug resistance has dramatically affected its clinical use. Therefore, novel treatment strategies are critical to improving patient outcomes. Herein, we investigated the ability of the epigenetic drug SAHA to increase the sensitivity of chemoresistant CRC cells to 5-FU. In addition, we evaluated the potential genotoxic risk of SAHA+5-FU combination treatment. As a model system, we used three CRC cell lines, HT-29, SW480, and HT-29/EGFP/FUR, differing in their resistance to 5-FU. CRC cell lines were exposed to sub-toxic SAHA concentrations for 24 h, followed by a 48 h treatment with 5-FU. The cytotoxicity of SAHA, 5-FU, and SAHA+5-FU was measured by the MTT test, the genotoxicity by the comet assay, and the micronucleus test. The apoptotic/necrotic activity was assessed using morphological criteria.

We found a synergic decrease in the viability of HT-29 and SW480 cells, but not the most resistant HT-29/EGFP/FUR cells after combined SAHA+5-FU exposure compared to 5-FU. Remarkably, SAHA most efficiently induced apoptosis in HT-29/EGFP/FUR cells compared to HT-29 and SW480 cells. Combined SAHA+5-FU treatment resulted in a synergistic increase in apoptotic/necrotic cells in HT-29 cell line, while rather additive/sub-additive effect was determined in the SW480 and HT-29/EGFP/FUR cells. At the same time, however, a synergistic rise in micronuclei was found in CRC cell lines (at least at some concentrations). We have shown that SAHA can sensitize CRC cells to 5-FU; therefore, epigenetic and convential drug combinations could be beneficial for the patients. However, the increase in micronucleus formation after combined SAHA+5-FU treatment indicates a potential health hazard. The clastogenic activity could contribute to cancer heterogeneity, favoring progeny of such aberrant cells to clonal expansion. Therefore, developing new specific epigenetic drugs or nanocarriers for targeted drug delivery might reduce the potential genotoxic risk.

1. Introduction

Despite improvements in diagnostic procedures and more effective therapeutic advances, colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide [1]. Chemotherapy coupled with surgery is the most common treatment form and, in the case of advanced metastatic CRC, the only way to increase the survival rate of such patients.

In most CRC cases, the first-line chemotherapeutic agent is 5-fluorouracil (5-FU), the antimetabolite drug [2]. Its therapeutic effect is mediated either by inhibiting the thymidylate synthase (TS) or by misincorporating 5-FU metabolites into DNA and RNA [3]. The enzymatic TS function is inactivated due to the formation of a stable complex with 5-FU metabolites, resulting in intracellular nucleotide pool perturbations required for DNA replication and repair. Moreover, incorporating 5-FU metabolites into DNA causes cell cycle arrest and apoptosis [4–6]. 5-FU metabolites are also incorporated into RNA, inducing rRNA maturation disorders and inhibiting pre-mRNA splicing. However, more than 80 % of 5-FU is catabolized to inactive metabolites in the liver and eliminated from the organism [4]. Combination 5-FU with other chemotherapeutics (*e.g.*, leucovorin, oxaliplatin, irinotecan), the monoclonal antibodies (*e.g.*, anti-EGFR or anti-VEGF) or immunotherapy has vastly improved clinical outcomes. Nevertheless, the increased adverse side effects and the development of drug resistance remain the principal causes of treatment failure [7]. Novel therapeutic strategies are, therefore, needed to improve patients` drug response rates.

Genomic profiling has improved understanding of the molecular

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pathogenesis of CRC. Epigenetic alterations, along with gene mutations, have been shown to play a critical role in the molecular CRC heterogeneity [8]. In addition to DNA methylation and non-coding RNAs, histone modification is increasingly recognized as a crucial mechanism underlying CRC development [9]. Covalent histone modification, controlled by histone deacetylases (HDACs) and histone acetyltransferases (HATs), affects the nucleosomal conformation and chromatin architecture, leading to changes in gene expression. The increased expression and activity of HDACs, frequently detected in tumor tissues [10] has been associated with poor prognosis of CRC patients [11]. Dysregulation of HDACs expression promotes oncogenic signaling by silencing tumor suppressor genes transcription or by the alteration of critical target genes expression regulating oncogenic pathways [10]. As histone modification is a reversible process, inhibiting the HDAC enzymes became a promising cancer therapeutic strategy for CRC [12].

Suberoylanilide hydroxamic acid (SAHA), also known as Vorinostat or Zolinza, is the first United States Food and Drug Administration (U.S. FDA)-approved HDAC inhibitor for the treatment of cutaneous T cell lymphoma [13]. The therapeutic effect of SAHA, a pan-HDAC inhibitor, is mediated by inhibiting HDACs due to direct binding to the zinc atom of the catalytic domain of these enzymes. SAHA, however, can affect gene transcription also indirectly by increased acetylation of proteins involved in cancer-relevant pathways, including transcription factors [14]. In preclinical studies, SAHA has inhibited cell proliferation, cell cycle, inflammation, angiogenesis, and induced apoptosis *in vitro* and *in vivo* [15]. Although HDAC inhibitors have failed as monotherapy in solid tumors due to limited effectiveness, adverse effects, and acquisition of drug resistance [16], several studies have shown their synergistic effect in combination with anticancer drugs [17]. SAHA combined with cisplatin has shown a synergistic antiproliferative effect in larynx cancer cells [18] and cholangiocarcinoma cells [19]. A strong synergistic antiproliferative effect between SAHA and 5-FU was determined in several human hepatoma cell lines [20], human squamous cancer cell lines [21], and colon cancer cell lines [22]. Moreover, combined SAHA+5-FU treatment resulted in the downregulation of TS protein in colon cancer xenograft models *in vivo* [23]. Regmi and colleagues have recently demonstrated that SAHA pretreatment overcomes 5-FU resistance in IFIT2-depleted oral squamous carcinoma cells *in vitro* and mouse xenografts *in vivo* [24].

Combining HDAC inhibitors with antitumor drugs may be a promising strategy to reverse chemoresistance, thus increasing the efficacy of conventional CRC chemotherapy. Therefore, the objective of our study was to evaluate the ability of SAHA to increase the susceptibility of the highly chemoresistant CRC cell lines to 5-FU. In addition, we assessed the potential hazard of such combined SAHA+5-FU treatment as both 5-FU and SAHA were shown to induce genotoxic effects *in vitro* and *in vivo* [25,26]. To our best knowledge, data dealing with the risk assessment of combination therapy involving the epigenetic drug and conventional one is entirely lacking. Genomic instability can contribute to clonal evolution and cancer heterogeneity resulting in developing a secondary malignancy late in life.

The potential genotoxic hazard of combination therapy has not been assessed thoroughly yet.

The CRC cell lines HT-29, SW480, and HT-29/EGFP/FUR, differing substantially in the resistance to 5-FU we employed as a model system in our study. We pretreated the cells with sub-toxic SAHA concentrations ($\leq$ IC20) for 24 h before exposure to 5-FU (<IC50) for 48 h. To assess the impact of the SAHA+5-FU combined treatment on genomic stability, we used the alkaline comet assay and the micronucleus test. Because of their robustness, sensitivity, and statistical power to evaluate DNA breakage, a hallmark of mutagenicity and carcinogenicity, they are the most frequently used assays in genetic toxicology. Finally, to objectively evaluate the benefit/potential risk of the combined SAHA+5-FU treatment, the combinatory factor (CF) was calculated. This parameter determines the synergism, additivity, or antagonism between SAHA and 5-FU at each measured endpoint.

2. Materials and methods

2.1. Chemicals

Ethidium bromide (EtBr, CAS number 1239-45-8), low-melting-point (LMP, CAS number 39346-81-1) agarose, normal-melting-point (NMP, CAS number 9012-36-6) agarose, Triton X-100 (CAS number 9002-93-1), HEPES (CAS number 7365-45-9), 5-fluorouracil (5-FU, CAS number 51-21-8), suberoylanilide hydroxamic acid (SAHA, CAS number 149647-78-9) were purchased from Sigma-Aldrich (Lambda Life, Slovakia), 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT, CAS Number 298-93-1) purchased from Sigma-Aldrich Chemia, Germany.

All other chemicals and solvents were of analytical grade from commercial suppliers.

2.2. Cell lines

Three human colon adenocarcinoma cell lines differing in their sensitivity to 5-FU were used in this study. The HT-29 cell line (ECACC no. 91072201), the most sensitive cell line to 5-FU, SW480 cells (ATCC® CCL-228™), with medium sensitivity to this drug and the resistant HT-29/EGFP/FUR line. The HT-29/EGFP/FUR cells, stably expressing the green fluorescent protein (EGFP) were developed by exposing parental chemonaive cell line HT-29/EGFP to gradually increasing concentrations of 5-FU, up to clinically relevant plasma concentrations of 2 μg.ml^{-1}, as described in [12]. All cell lines were maintained in high glucose (4.5 g.l^{-1}) Dulbecco's modified Eagle medium (DMEM, Gibco, Lamba Life, Slovakia) supplemented with 10 % fetal calf serum (FCS; Biochrom AG, Germany), 1 % GlutaMAX (Gibco by Life Technologies, USA), and addition of 0.25 % gentamicin (Sandoz, Germany). In the case of HT-29/EGFP/FUR cells, 5-FU at a concentration of 2 μg.ml^{-1} was added to the medium. The cells were cultivated at 37 °C, in a humidified atmosphere of 5 % CO_2.

2.3. Cell treatment

The exponentially growing cells were exposed to selected concentrations of SAHA and 5-FU depending on their sensitivity to particular drug for 24 h–72 h. The working concentrations of SAHA: 0.16 g.ml^{-1}; 0.21 g.ml^{-1}; 0.26 g.ml^{-1} (for HT-29 cells); 0.32 g.ml^{-1}; 0.37 g.ml^{-1}; 0.42 g.ml^{-1} (for SW 480 cells) and 0.40 g.ml^{-1}; 0.53 g.ml^{-1}; 0.66 g.ml^{-1} (for HT 29/EGFP/FUR cells) were prepared freshly before use from the stock solution 5 mM diluted in culture media. The working concentrations of 5-FU: 0.15 g.ml^{-1}; 0.23 g.ml^{-1}; 0.3 g.ml^{-1} (HT-29 cells); 0.9 g.ml^{-1}; 1.2 g.ml^{-1}; 1.8 g.ml^{-1} (SW 480 cells); 20.2 g.ml^{-1}; 30.2 g.ml^{-1}; 40.2 g.ml^{-1} (HT-29/ EGFP/FUR cells) from the stock solution 1 mg/mL diluted in culture media.

In the case of combined treatment, cells were pretreated with SAHA for 24 h, followed with 48 h exposure to 5-FU.

The treatment of the cells was finished by removing the medium with drugs and washing the cells twice with phosphate-buffered saline (PBS). Then, the cells were either processed immediately or incubated in a fresh medium during different time intervals and then processed.

2.4. Cell viability assay

The MTT assay was based on the protocol described by Mosmann [50], with minor modifications. In brief, the cells were plated into a 96-well plate at a density of 1.5×10^4 cells /well. After reaching a 75 % confluence, the cells were treated with different concentrations of 5-FU or SAHA for 24–72 h or exposed to SAHA+5-FU. The photometric evaluation (at 540-nm excitation and 690-nm emission wavelengths) was carried out using the Bio-Rad x MarkTM microplate spectrophotometer and Microplate Manager 6 software (Bio-Rad, Czech Republic).

IC50 values were calculated from the dose-response curves using

CalcuSyn software (Biosoft, Cambridge, UK).

2.5. Single-cell gel electrophoresis (SCGE)

To evaluate the capacity of SAHA, 5-FU, and SAHA+5-FU to induce DNA strand breaks, we used the alkaline comet assay [52,54]. In brief, cells (1.2×10^5) were gently resuspended and mixed in 1 % LMP agarose in PBS (Ca^{2+} and Mg^{2+} free); the final concentration of LMP was 0.75 %. Next, 50 μL of this cell suspension was dropped on a 1 % NMP agarose pre-coated slide and covered by a coverslip. After solidification of gel, slides were placed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-HCl, pH = 10 and 1 % Triton X-100, at 4 °C) for 60 min to remove cellular proteins. After lysis, slides were transferred to a horizontal electrophoretic box and immersed in an alkaline electrophoretic solution (300 mM NaOH, 1 mM Na2EDTA, pH > 13). After 30 min unwinding time, a voltage of 19 V (0.8 V/cm and 300−350 mA) was applied for 20 min at 4 °C. The slides were neutralized with 3 × 5 min washes with Tris–HCl (0.4 M, pH 7.4) and dried with ethanol. Before scoring, slides were stained with ethidium bromide (EtBr, 5 $\mu g.ml^{-1}$, 20 μL /slide). EtBr-stained nucleoids were examined with Zeiss Axio Imager.Z2 fluorescence microscope (Zeiss, Germany) using the computerized image analysis (Metafer 3.6, MetaSystems GmbH, Altlussheim, Germany). The percentage of DNA in the tail (% of tail DNA) was used as a parameter of DNA damage measurement [53]. We used 3 parallels for each concentration and evaluated 150 nucleoids per gel in each electrophoretic run.

2.6. Micronucleus test

Cells at a density of 1.5×10^5 cells were seeded on Petri dish (Ø = 60 mm). Next day, cells were treated with SAHA, 5-FU or SAHA+5-FU as described above. After the treatment, cells were fixed with the ice-cold fixative solution for 15 min, washed with distilled water, and dried overnight. Cells were stained with DAPI (0.2 $\mu g.ml^{-1}$) in Mc'Ilvaine staining solution. Micronuclei (MNi) were identified according to the criteria specified by Miller et al. [27]. The proliferation status (mitotic index, MI) of the human CRC cells was measured according to Eckl and Raffelsberger [28]; cell death (apoptosis and necrosis) was determined using morphological criteria (fragmentation of nuclei) described by Oberhammer et al. [29]. Two thousand cells per dish were analyzed using the fluorescence microscope Olympus BX51 (Olympus Optical CO, Ltd., UK). Data are presented as means ± S.D. of two parallel dishes per sample from at least two independent experiments.

2.7. Combinatory factor

The combinatory factor (CF) values were calculated to determine the type of interaction between SAHA and 5-FU. We used the formula described by Surralles et al. [51]:

$$CF = \frac{MN_{B+D} - MN_C}{(MN_B - MN_C) + (MN_D - MN_C)}$$

where MN_C is the number of micronuclei (MNi) in the control, MN_{B+D} is the number of MNi in the cultures exposed to SAHA+5-FU, MN_B is the number of MNi in cultures exposed to SAHA alone, and MN_D is the number of MNi in cultures treated with 5-FU alone. A value of Cf > 1 indicates synergism; Cf = 1 indicates additivity, and Cf < 1 indicates antagonism.

2.8. Statistical analysis

Data are given as mean ± S.D. from at least three independent experiments with triplicates per sample. The differences between treated samples and untreated control were evaluated by the Student's *t*-test and one-way analysis of variance (ANOVA). The threshold of statistical significance was set to $p < 0.05$.

3. Results

3.1. SAHA increased the susceptibility of HT-29, SW480, and HT-29/EGFP/FUR cells to 5-FU

The preliminary experiments focused on selecting suitable SAHA and 5-FU concentrations and exposure time for combined SAHA+5-FU treatment. HT-29, SW480, and HT-29/EGFP/FUR cells were exposed to various concentrations of individual drugs for 24 h, 48 h, and 72 h, and their viability was assessed by the MTT assay. IC50 values for SAHA and 5-FU for a particular cell line and exposure time calculated from the dose-response curves are shown in Table 1. The cell survival after SAHA treatment correlated with the 5-FU resistance determined in individual cell lines. HT-29 cells were most sensitive to SAHA and 5-FU, while the 5-FU-resistant HT-29/EGFP/FUR cells were also the most resistant to SAHA. SW480 cells showed intermediate sensitivity to 5-FU and SAHA compared to HT-29 and HT-29/EGFP/FUR cells.

As SAHA downregulated TS expression within 24 h [30], the sequential treatment was selected, in which the pretreatment with SAHA for 24 h followed by 48 h exposure to 5-FU. For the combined SAHA+5-FU treatment, three concentrations of SAHA and 5-FU were selected for each cell line, the toxicity of which never exceeded 50 %. However, due to large differences in sensitivity to 5-FU and also to SAHA, equivalent concentrations, for at least SAHA, for all three cell lines could not be used.

In HT-29 cells, the selected SAHA concentrations affected cell survival only negligibly; cell viability ranged between 100 to 93 %, while the cell survival after exposure to 5-FU was not reduced by more than 30 % at the highest concentration (Fig. S1A). In combined treatment, SAHA showed a variable effect on the survival of 5-FU-treated HT-29 cells. A synergistic reduction of cell viability (CF > 1) was determined at all SAHA concentrations in combination with the lowest 0.15 $\mu g.ml^{-1}$ 5-FU concentration while at the higher, 0.23 $\mu g.ml^{-1}$ and 0.3 $\mu g.ml^{-1}$, 5-FU concentrations, an additive effect was observed (Fig. S1A).

SAHA also enhanced the sensitivity of SW480 cells to 5-FU (Fig. S1B). The synergistic cytotoxic effect was detected at two lower 5-FU concentrations (0.9 $\mu g.ml^{-1}$, 1.2 $\mu g.ml^{-1}$) except one SAHA+5-FU combination (0.42 $\mu g.ml^{-1}$+1.2 $\mu g.ml^{-1}$). Although SAHA inhibited the cell viability at the highest 1.8 $\mu g.ml^{-1}$ 5-FU concentration, only an additive effect was reached. A dose-dependent reduction in cell survival after combined SAHA+5-FU treatment was also detected in HT-29/EGFP/FUR cells (Fig. S1C). However, in contrast to HT-29 and SW480 cells, no synergism between SAHA and 5-FU was detected at any SAHA and 5-FU concentrations.

Further experiments were aimed to investigate the impact of combined SAHA+5-FU treatment on the DNA breakage, mitotic activity, micronucleus formation, and induction of apoptosis in CRC cell lines.

3.2. SAHA pretreatment did not affect the level of DNA strand breaks induced by 5-FU

The comet assay (also known as the single cell gel electrophoresis) has been w used as a first-line method to detect DNA strand breaks, a standard biomarker of DNA damage, induced by genotoxic agents [31]. A significant increase in the level of DNA strand breaks was detected in all CRC cell lines after exposure to SAHA and 5-FU alone, but the growth of DNA damage was not dose-dependent (Table 2). Interestingly, SAHA induced the highest level of DNA strand breaks in HT-29/EGFP/FUR cells (a nearly 3-fold increase compared to control cells), while an almost equal level of DNA breakage (approximately 2-fold rise) was found in HT-29 and SW480 cells. On the other hand, 5-FU was the most efficient in SW480 cells compared to HT-29 and HT-29/EGFP/FUR cells; a nearly 4-fold rise of DNA damage was observed in this cell line. Interestingly, combined SAHA+5-FU treatment did not result in

Table 1
IC50 values of SAHA and 5-FU in HT-29, SW480, and HT-29/EGFP/FUR cell lines after 24 h, 48 h, 72 h of treatment.

		IC50 [μg. ml^{-1}]				IC50 [μg. ml^{-1}]		
Cell line	Agent	24 h	48 h	72 h	Agent	24 h	48 h	72 h
HT-29	5-FU	1.07	0.56	0.48	SAHA	0.43	0.44	0.30
SW480		4.64	2.30	2.27		0.64	0.58	0.78
HT-29/EGFP/FUR		74.64	76.48	57.71		0.97	0.95	0.34

Table 2
The percentage of DNA in tail detected in HT-29, SW480, and HT-29/EGFP/FUR cell after exposure to SAHA [24 h], 5-FU [48 h], and combined treatment SAHA + 5-FU [24 h + 48 h].

	HT-29 cells			SW480 cells			HT-29/EGFP/FUR cells	
SAHA [μg. ml^{-1}]	5-FU [μg. ml^{-1}]	DNA in tail [%]	SAHA [μg. ml^{-1}]	5-FU [μg. ml^{-1}]	DNA in tail [%]	SAHA [μg. ml^{-1}]	5-FU [μg. ml^{-1}]	DNA in tail [%]
Control	–	4.10 ± 0.09	Control		8.78 ± 1.08	Control		3.87 ± 0.48
	0.15	13.87 ± 0.85***		0.9	22.98 ± 1.36***		20.2	12.20 ± 0.19**
	0.23	14.27 ± 0.99***		1.2	24.24 ± 1.93***		30.2	12.06 ± 0.19**
	0.30	12.97 ± 0.62***		1.8	23.89 ± 1.64***		40.2	12.68 ± 1.27**
0.16		6.04 ± 0.73*	0.32		11.18 ± 0.77*	0.40		11.68 ± 1.64
0.21		6.78 ± 0.85*	0.37		12.77 ± 0.54*	0.53		11.73 ± 1.16*
0.26		5.81 ± 1.24	0.42		9.72 ± 2.62	0.66		12.83 ± 1.01**
	0.15	10.89 ± 1.08**		0.9	19.58 ± 1.45***		20.2	11.84 ± 1.64**
0.16	0.23	13.74 ± 1.17**	0.32	1.2	20.85 ± 0.77***	0.40	30.2	15.05 ± 1.10**
	0.30	16.45 ± 0.24***		1.8	18.67 ± 1.49**		40.2	18.96 ± 1.03***
	0.15	14.43 ± 0.95**		0.9	26.93 ± 1.31***		20.2	18.38 ± 1.32***
0.21	0.23	18.58 ± 0.75***	0.37	1.2	29.32 ± 1.65***	0.53	30.2	14.23 ± 0.59**
	0.30	15.79 ± 0.66***		1.8	27.37 ± 2.16***		40.2	12.97 ± 0.88**
	0.15	15.11 ± 0.02***		0.9	23.94 ± 1.03***		20.2	14.00 ± 0.64**
0.26	0.23	17.15 ± 0.63***	0.42	1.2	25.49 ± 0.83***	0.66	30.2	14.45 ± 0.50**
	0.30	16.54 ± 0.31***		1.8	19.59 ± 0.74***		40.2	17.17 ± 0.58***

Data represent the mean ± S.D. from at least 3 independent experiments with 3 parallels per sample. A total of 150 nucleoids were scored per each sample in one electrophoretic run. Significantly different from control.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.01$.

additional growth of DNA damage in exposed cells; the levels of DNA damage were comparable with those produced by 5-FU alone, indicating an antagonistic effect. However, it cannot be excluded that the highly damaged cells were lost already during the lysis. DNA fragments produced during apoptosis are much too small to be detected by the comet assay; they can diffuse away from the gel during lysis [32]. The CF values determined for each SAHA+5-FU combination supported our suggestion.

3.3. Combined SAHA+5-FU treatment increased synergistically the level of micronuclei in CRC cells

Micronucleus formation indicates chromosomal instability; therefore, its assessment has become an important endpoint in genotoxicity studies. The advantage of the micronucleus test is that, in addition to genotoxic effects, it provides information on the mitotic and apoptotic activity of exposed cells. [34].

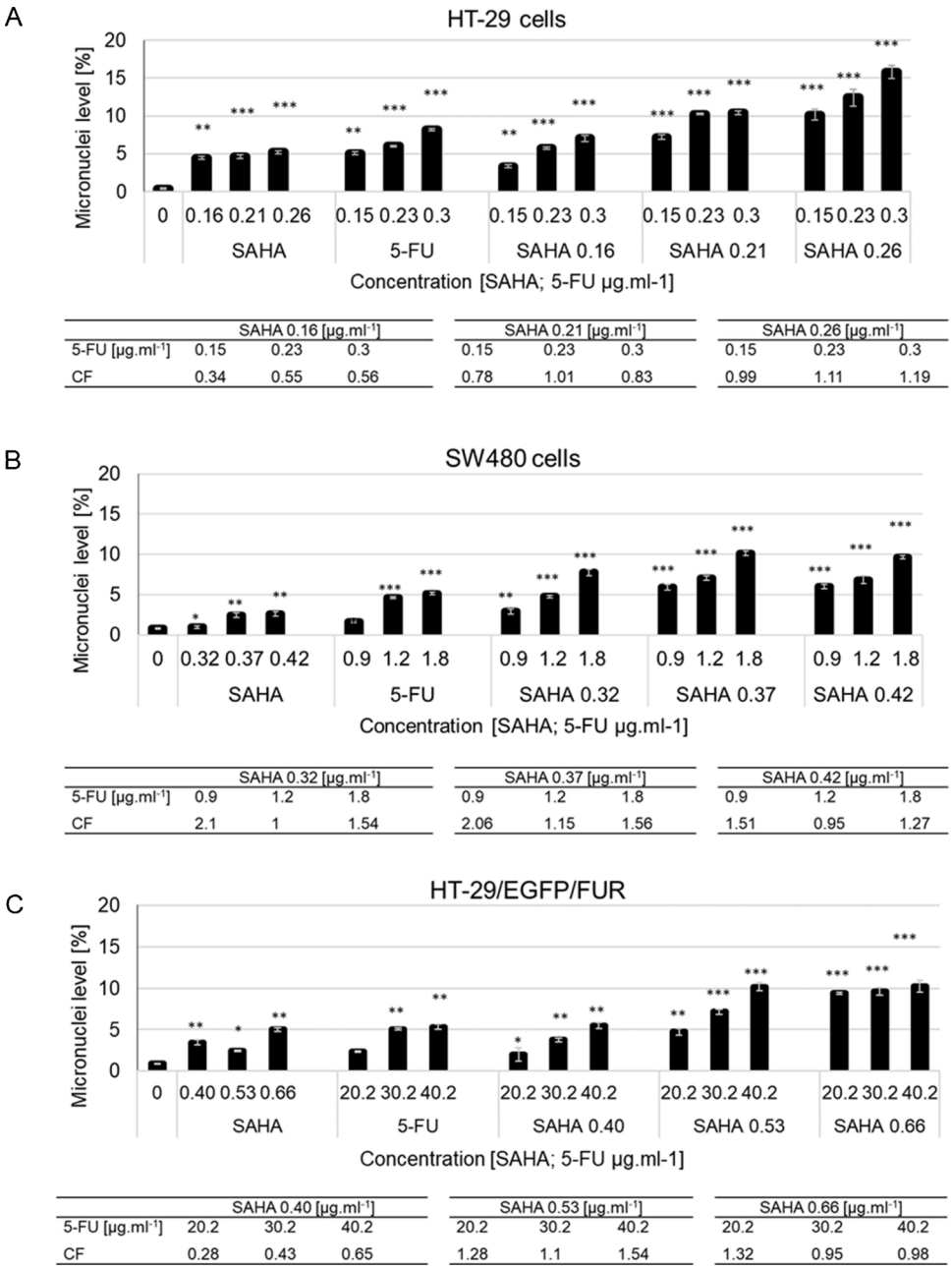
In general, cell exposure to SAHA or 5-FU alone significantly inhibited the proliferation activity in all CRC cell lines, though the effect of SAHA was less pronounced in SW480 cells (Tab. S1). The strongest antiproliferative activity of combined SAHA+5-FU was detected in HT-29 cells at the highest 0.26 μg.ml^{-1} SAHA concentration.

SAHA and 5-FU alone significantly increased the MNi level in each cell line regardless of its chemoresistance to this antimetabolite drug. In HT-29 cells, SAHA induced almost the same MNi number at each concentration, while a linear dose-dependent MNi growth ($R^2 = 0.925$) was detected in 5-FU-treated cells (Fig. 1A). The combined SAHA+5-FU treatment showed an antagonistic effect at the lowest 0.16 μg.ml^{-1} SAHA concentration. The MNi number was lower or approximately equal to that induced by 5-FU alone. In contrast, a sub-additive to an additive effect on the level of MNi was found at the 0.21 μg.ml^{-1} SAHA concentration, and at the highest 0.26 μg.ml^{-1} SAHA concentration additivity or synergism between SAHA and 5-FU was observed. A linear dose-dependent rise of MNi was detected in SW480 cells (Fig. 1B) after exposure to SAHA and 5-FU alone ($R^2 = 0.8672$ and 0.963, respectively). Interestingly, combined SAHA+5-FU treatment caused either synergistic or additive rise of MNi at all combinations of concentrations in this cell line. In HT-29/EGFP/FUR cells, SAHA and 5-FU alone significantly increased the level of MNi, although the effect was not dose-dependent (Fig. 1C). Combined SAHA+5-FU treatment resulted in a strong antagonistic effect on the MNi level at the lowest 0.40 μg.ml^{-1} SAHA concentration, while an additive to synergistic effect was detected at the higher SAHA concentrations (0.53 μg.ml^{-1} and 0.66 μg.ml^{-1}).

The basal level of apoptotic/necrotic cells in individual cell lines was comparable (Table 3). Interestingly, SAHA induced the highest number of apoptotic/necrotic cells in the chemoresistant HT-29/EGFP/FUR cells (a 3-10-fold increase compared to control). On the other hand, the slightest effect was detected in HT-29 cells (a 2-3-fold increase compared to control). Approximately similar levels of apoptosis/necrosis were found in SW480 cells after exposure to SAHA and 5-FU (a 2-5-fold increase compared to control cells). A synergistic effect of combined SAHA+5-FU on the apoptosis/necrosis level (Cf>1) was detected only in HT-29 cells at all SAHA+5-FU combinations except one (SAHA+5-FU: 0.16 μg.ml^{-1} + 0.15 μg.ml^{-1}). On the other hand, antagonism was determined between SAHA and 5-FU in SW480 and HT-29/EGFP/FUR cells which might be caused by the loss of highly damaged cells from the culture during cell processing.

4. Discussion

CRC cell lines differing in intrinsic or acquired resistance to conventional drugs are valuable preclinical *in vitro* models for studying the



	SAHA 0.16 [µg.ml^{-1}]			SAHA 0.21 [µg.ml^{-1}]			SAHA 0.26 [µg.ml^{-1}]		
5-FU [µg.ml^{-1}]	0.15	0.23	0.3	0.15	0.23	0.3	0.15	0.23	0.3
CF	0.34	0.55	0.56	0.78	1.01	0.83	0.99	1.11	1.19

	SAHA 0.32 [µg.ml^{-1}]			SAHA 0.37 [µg.ml^{-1}]			SAHA 0.42 [µg.ml^{-1}]		
5-FU [µg.ml^{-1}]	0.9	1.2	1.8	0.9	1.2	1.8	0.9	1.2	1.8
CF	2.1	1	1.54	2.06	1.15	1.56	1.51	0.95	1.27

	SAHA 0.40 [µg.ml^{-1}]			SAHA 0.53 [µg.ml^{-1}]			SAHA 0.66 [µg.ml^{-1}]		
5-FU [µg.ml^{-1}]	20.2	30.2	40.2	20.2	30.2	40.2	20.2	30.2	40.2
CF	0.28	0.43	0.65	1.28	1.1	1.54	1.32	0.95	0.98

Fig. 1. Micronuclei level evaluated in HT-29 [A|, SW480 [B] and HT-29/EGFP/FUR [C] after SAHA [24 h], 5-FU [48 h] and combinated treatment SAHA+5-FU [24 h + 48 h]. Combinatory factor [CF] values are listed in tables below each graph [CF < 1 antagonism; CF = 1 additivity; CF > 1 synergism]. Data represent the mean values ± SD from at least 3 independent experiments; at least 2000 cells were analyzed per sample, two parallel plates *per* sample in each experiment. Significantly different from control *p < 0.05, **p < 0.01, ***p < 0.001.

efficacy and safety of combination therapy. To objectively evaluate the ability of SAHA to overcome chemoresistance, we selected three CRC cell lines differing in sensitivity to 5-FU. In contrast to 5-FU resistance, all CRC cell lines were almost equally sensitive to SAHA, suggesting their high susceptibility to this pan-HDAC inhibitor. The significant increase in apoptotic/necrotic cells indicated that SAHA triggers apoptosis in these cells either by restoring tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or up-regulating pro-apoptotic protein expressions such as Bim, Bak, and Bax [15]. Apart from the pro-apoptotic activity, we simultaneously observed proliferation inhibition and genomic instability induction in all SAHA-exposed CRC cell lines. In line with our results, SAHA showed clastogenic activity in Chinese hamster ovary (CHO) cells but not in normal human lymphocytes [40]. The increased sensitivity of cancer cells to HDAC inhibition is explained by overexpression of a specific HDAC isoform or group of HDACs, which render them more sensitive to the inhibition than normal tissue cells [10]. The enhanced level of DNA strand breaks and MNi could be caused by blocking the DNA replication forks and activating dormant replication origins in cancer cells [39]. A recent study has revealed that repeated administration of SAHA using clinically relevant doses caused structural chromosomal damage and numerical chromosomal abnormalities, DNA hypomethylation, and apoptosis *in vivo*. In addition, SAHA altered the expression of many genes involved in DNA damage/repair pathways [41]. The genotoxic effects induced by SAHA could be tolerated to some extent in the case of life-threatening diseases, such as cancer. However, in combination with conventional drugs such as 5-FU, which also has DNA damaging and clastogenic properties [42], the genetic changes might accumulate within the cells and contribute to undesired properties leading to secondary malignancies. Therefore, a careful toxicological evaluation of such combination therapy is required prior to clinical use.

SAHA pretreatment, followed by exposure to 5-FU, synergistically reduced the viability of HT-29 and SW480 cells, while antagonism was found in the highly chemoresistant HT-29/EGFP/FUR cells. In line with

Table 3
The percentage of apoptotic/necrotic cells detected in HT-29, SW480 and HT-29/EGFP/FUR cells after exposure to SAHA [24 h], 5-FU [48 h], and combined treatment SAHA+5-FU [24 h + 48 h].

	HT-29 cells				SW480 cells				HT-29/EGFP/FUR cells		
SAHA [μg. ml^{-1}]	5-FU [μg. ml^{-1}]	Apoptosis/ necrosis [%]	CF	SAHA [μg. ml^{-1}]	5-FU [μg. ml^{-1}]	Apoptosis/ necrosis [%]	CF	SAHA [μg. ml^{-1}]	5-FU [μg. ml^{-1}]	Apoptosis/ necrosis [%]	CF
Control	–	2.20 ± 0.85	–	Control		3.4 ± 0.97		Control		2.95 ± 0.75	
	0.15	3.20 ± 0.07			0.9	8.10 ± 0.71			20.2	3.25 ± 0.18	
	0.23	6.45 ± 0.32			1.2	11.85 ± 0.18			30.2	4.65 ± 0.88	
	0.30	7.25 ± 0.46			1.8	15.40 ± 0.85			40.2	6.00 ± 0.35	
0.16		5.02 ± 0.64		0.32		5.61 ± 0.45		0.40		9.15 ± 0.49	
0.21		5.96 ± 0.64		0.37		10.60 ± 0.23		0.53		21.95 ± 0.18	
0.26		7.83 ± 0.85		0.42		15.05 ± 1.03		0.66		30.5 ± 1.63	
	0.15	6.40 ± 0.82	0.97		0.9	9.10 ± 0.96	0.79		20.2	8.55 ± 0.77	0.72
0.16	0.23	14.25 ± 1.57	1.59	0.32	1.2	11.80 ± 1.43	0.76	0.40	30.2	12.60 ± 2.50	**1.05**
	0.30	13.64 ± 1.80	1.37		1.8	14.00 ± 2.96	0.73		40.2	15.15 ± 1.02	**1.16**
	0.15	13.65 ± 2.00	2.18		0.9	12.30 ± 1.40	0.73		20.2	13.35 ± 0.55	0.50
0.21	0.23	16.00 ± 4.11	1.62	0.37	1.2	15.25 ± 2.17	0.74	0.53	30.2	10.50 ± 1.76	0.34
	0.30	16.45 ± 1.80	1.53		1.8	17.35 ± 0.48	0.71		40.2	14.60 ± 1.18	0.50
	0.15	16.20 ± 0.59	1.96		0.9	12.95 ± 4.14	0.57		20.2	15.30 ± 0.53	0.42
0.26	0.23	16.15 ± 2.08	1.34	0.42	1.2	15.90 ± 1.83	0.61	0.66	30.2	10.5 ± 1.76	0.46
	0.30	20.65 ± 2.27	1.65		1.8	17.70 ± 0.48	0.59		40.2	25.40 ± 2.59	0.70

The data represent the mean values ± SD from at least 3 independent experiments; at least 2000 cells were analyzed per sample, two parallel plates per sample in each experiment. CF – combinatory factor, CF < 1 antagonism; CF = 1 additivity; CF > 1 synergism.

our results, Hosokawa et al. [37] reported a synergistic cytotoxic effect of SAHA+5-FU in SW480 cells. On the other hand, Ikehata et al. [43] determined an antagonistic effect if a simultaneous SAHA+5-FU exposure was employed. These conflicting results highlighted the importance of a treatment schedule for effective combination cancer chemotherapy. In addition, combined SAHA+5-FU treatment induced a synergistic pro-apoptotic effect in HT-29 cells, but antagonism was found in SW480 and HT-29/EGFP/FUR cells. We suppose that the antagonism might be caused by the loss of highly damaged cells during cell processing prior to scoring because SAHA and 5-FU themselves significantly increased the apoptosis in these two CRC cells. Synergism between SAHA and 5-FU to induce apoptosis was also detected in tumor skin cells [44] and squamous cancer cells [21]. Notably, phase I clinical trial of combined SAHA and 5-FU treatment brought promising results [46]. However, a randomized phase II clinical study failed because the intratumor TS level was not reduced [47].

Unfortunately, combined SAHA+5-FU treatment resulted in a synergistically increased level of MNi in all CRC cell lines, even if we did not detect an enhanced level of DNA strand breaks compared to that produced by SAHA or 5-FU alone. Despite the high sensitivity, the comet assay cannot detect highly damaged DNA or DNA fragments produced during apoptosis because such too small DNA pieces diffuse away from the gel already during lysis [32]. Micronucleus formation contributes to malignant cell transformation due to the gain or loss of genetic material [45]. Progeny of such micronucleated cells may possess aberrant phenotypic differences compared to the parental cells, favoring them to clonal expansion and possibly accelerating the transformation process [33]. Therefore, the increased clastogenic effect of combined SAHA+5-FU treatment might indicate potential health hazards. To our best knowledge, it is the first study dealing with the potential health hazard of combination therapy involving epigenetic and conventional drugs.

In conclusion, we showed that SAHA was able to sensitize the chemoresistant CRC cells to 5-FU efficiently by activating the process of apoptosis and inhibition of proliferation in all CRC cells. Remarkably, SAHA was most efficient in HT-29/EGFP/FUR cells which showed more than 135-fold higher IC50 value for 5-FU than HT-29 cells. However, despite these promising results, combined SAHA+5-FU treatment resulted in synergistic clastogenic effects in all cell lines, which might contribute to tumor heterogeneity or develop a clonal expansion. Therefore, it is desirable to develop new selective HDAC inhibitors that target specific HDACs, overexpressed and associated with poor cancer patient outcomes. A promising strategy could be the employment of nanocarriers that protect cancer drugs from first-pass metabolism and early enzymatic degradation in the gastrointestinal tract and ensure an adequate supply of the drug(s) to the target tissue [49].

Data availability

Raw data and other supplementary material are available at the following repository: osf.io/7zpcj.

No data was used for the research described in the article.

Data will be made available on request.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mrgentox.2022.503445.

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