

5-Aza Cytidine Sensitizes HCT116 Colorectal Cancer Cells to 5-Fluorouracil: Enhanced Efficacy and Altered Genomic Variation

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Abstract: The escalating global burden of cancer, with a near 40% mortality increase over four decades and a projected 60% surge this decade, culminating in an estimated 13 million deaths by 2030, underscores the urgent need for improved therapies. Colorectal cancer (CRC), the third most frequent malignancy and second leading cause of cancer mortality worldwide, accounting for approximately 10% of diagnoses, necessitates innovative treatment strategies. This *in vitro* study investigated the effects of combined exposure to 5-fluorouracil (5-FU) and 5-Aza cytidine (5-Aza) on human colorectal carcinoma cells (HCT116). Employing MTT, wound healing, and colony formation assays, we observed significant reductions in cell viability, migration, and proliferation with the combined treatment compared to monotherapies. Whole-exome sequencing (WES) revealed a substantial decrease in the number and diversity of single-nucleotide polymorphisms (SNPs) in 5-FU-treated cells. These findings demonstrate that combined 5-Aza/5-FU exposure alters HCT116 cellular responses; however, formal pharmacological synergy cannot be inferred from the present experimental design. The synergistic effect of 5-Aza sensitized CRC cells to 5-FU.

Keywords: CRC, 5-FU, 5-Aza, WES, HCT116.

INTRODUCTION

The mortality rate of cancer has globally increased by almost 40% over the past four decades. It is expected that it will grow to reach 60% in the current decade, with an estimated death of 13 million cancer patients by 2030 [1]. Colorectal cancer (CRC) is an issue of public health, where it is considered the third most frequent type of cancer and the second leading cause of cancer-related deaths, for both sexes, in the world [2]. CRC accounts for 10% of the most commonly diagnosed types of cancer following breast and lung cancers, with a mortality rate of 9.4% among all types of cancer [3]. Etiologies include smoking, bad diet, obesity, and low physical activity [4]. Nevertheless, CRC can be genetically inherited [5].

CRC can develop as the inner linings of the colon or rectum grow to form polyps. Polyps are an abnormal growth of the colon mucosal tissue with a size of less than 1 cm and can evolve to create an early adenoma

[6]. The early adenoma can turn into an advanced adenoma, which can then develop into carcinoma [7]. However, only 10-15% of the advanced adenomas can develop into CRC.

5-Fluorouracil (5-FU) has been an essential component of systemic chemotherapy for the palliative and adjuvant treatment of CRC for over fifty years. Resistance to 5-FU is a significant barrier to the successful treatment of CRC and is associated with an increased risk of recurrence [8]. Therefore, several modulation techniques, including the deployment of 5-FU-based combination regimens and 5-FU pro-drugs, have been proposed and evaluated to boost the antitumor activity of this drug and overcome clinical resistance.

Despite the hopeful advances in CRC therapy, patient response rates to treatment continue to be poor. The benefit of 5-FU-based therapy is typically hampered by chemoresistance [9-11], which limits its clinical use [12, 13]. The pharmacological impact of 5-FU is regulated by genetic variations and dosing regimens [14], which might explain its resistance in one cell line but not the other.

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Due to the presence of cancer stem cell-like cells, cancer recurrence, metastasis, and angiogenesis, overcoming 5-FU chemoresistance is difficult [13]. However, the implementation of combination therapy might help. 5-Aza, a pyrimidine analog that demethylates the genome and reactivates transcriptionally silent genes [15], and arrests the cell cycle [16] is used. Earlier studies have proposed 5-Aza as neoadjuvant therapy for different cancers [17, 18] including CRC. It is indicated that the chemoresistance of 5-FU in CRC is associated with aberrant hypermethylation of several genes via DNMT1/DNMT3B [19]. Earlier studies have addressed the role of 5-Aza in regaining the chemosensitivity of CRC cells to 5-FU using 5-Aza -2'-deoxycytidine (DAC), where nontoxic levels of this drug prove successful in inducing apoptosis and restoring chemosensitivity to 5-FU [20].

Studies indicated the involvement of several methylated genes in the recurrence and chemoresistance to 5-FU. These genes (CCNE1, CCNDBP1, PON3, DDX43, and CHL) are associated with 5-Aza-mediated sensitization of CRC to 5-FU [8]; thus, they could serve as molecular targets.

Although aberrant DNA methylation has been implicated in 5-FU resistance and previous studies have investigated DNA-demethylating approaches for restoring fluoropyrimidine sensitivity, the phenotypic response of HCT116 colorectal cancer cells to combined 5-Aza cytidine and 5-FU exposure remains incompletely characterized. In particular, evidence integrating short-term metabolic viability with wound-closure and clonogenic endpoints under the same experimental framework is limited. Therefore, the present study investigated whether exposure to 5-Aza modifies the response of HCT116 cells to 5-FU by evaluating cellular viability, wound closure, and colony-forming capacity, with HEK293 cells included as a non-colorectal comparator. The study was designed as an exploratory *in vitro* assessment of the combination rather than a definitive demonstration of pharmacological synergy.

The present study evaluated the demethylating agent, 5-Aza, in restoring the chemosensitivity of CRC to 5-FU.

MATERIALS AND METHODS

Cell Line Maintenance

The human colorectal carcinoma cell line (HCT116, ATCC CCL-247) and the human embryonic kidney cell line (HEK293, ATCC CRL-1573) were cultured in

Dulbecco's Modified Eagle Medium (DMEM) (Gibco 41965-039, UK) supplemented with 10% of Foetal Bovine Serum (FBS), (Gibco A3160801, Germany) and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin (Gibco 15140-122, USA) in addition to 1% Non-Essential Amino Acids (NEAA) (Gibco 11140050, USA) and L-Glutamine (Gibco A29168-01, USA). Both cell lines were incubated at 37° C under 5% CO₂ (BINDER, USA). Cells were passaged weekly according to the standard laboratory conditions using 0.25% Trypsin-EDTA solution (Gibco 25200-056, Canada).

HCT116 was selected as an established human colorectal carcinoma model for evaluating responses to 5-FU-based treatment. HEK293 cells were included as a non-colorectal comparator to provide an exploratory assessment of treatment responses in a cell line of different tissue origin. HEK293 cells were not considered a tissue-matched normal colorectal epithelial control, and comparisons between HCT116 and HEK293 therefore should not be interpreted as demonstrating colorectal cancer-specific selectivity.

Chemotherapeutic Drugs

Both 5-Aza cytidine (5-Aza) and 5-Fluorouracil were purchased from Santa Cruz Biotechnology (USA). Stocks of 5 µM for 5-Aza and 50 µM for 5-FU were prepared in Dimethyl Sulfoxide (DMSO) (Thermo Scientific, USA) as a solvent and used to treat HCT116 and HEK293 cell lines with a final concentration of 5 µM. Drugs were applied solely or as neoadjuvant treatments in both cell lines. DMSO was also used as a treatment since it has known cytotoxic effects. Gradient concentrations of DMSO were used to assess the less cytotoxic concentration: 0.1%, 0.2%, 0.4%, and 0.8%.

Cell Count

Treated and untreated cells were harvested in 3 mL of media. Trypan Blue assay assessed the number of viable cells in each cell line. Briefly, 10 µL of each cell line suspension was mixed with 10 µL of trypan blue stain (0.4%) (Gibco 15250-061, USA) in a 1.5 mL tube. Then, 10 µL of each tube was applied to each side of a disposable Countess cell counting chamber slide (Invitrogen 100078809, USA), and both sides of the slides were inserted into the Countess II Automated Cell Counter (Invitrogen A27977, USA).

Cell viability assay (MTT)

HCT116 (passage 14) and HEK293 (passage 100) cells were seeded at 4.01×10^6 and 4.81×10^5 ,

respectively. Upon reaching 70% confluent monolayer, the media was discarded, and cells were treated with a concentration of 5 μ M of 5-Aza, 5 μ M of 5-FU, and for combination treatment, cells received 2.5 μ M 5-Aza plus 2.5 μ M 5-FU (total nominal drug concentration, 5 μ M). Monotherapy groups received 5 μ M of either 5-Aza or 5-FU. This fixed-total-concentration design was used for exploratory comparison of treatment conditions and was not designed to quantify pharmacological synergy or additivity. The plates were incubated for 72 h. The MTT assay was performed at 24 h, 48 h, and 72 h. After reaching each time point, the media in all wells were discarded, followed by washing with 100 μ L of PBS. PBS was discarded, 90 μ L of fresh media was added to all wells, followed by 10 μ L of the MTT reagent. After 4 hours of incubation at 37° C and 5% CO₂, the plate was centrifuged (Eppendorf 5810 R) at 1000 rpm for 40 seconds, and the media was removed. The MTT reaction was stopped by adding 100 μ L of filtered dimethyl sulfoxide (DMSO) (Thermo Scientific 20688, France), and the absorbance was measured at 570 nm using a microplate reader.

Each treatment condition was assessed in 3 technical replicate wells per experiment, and the experiment was independently repeated 3 times using separately cultured cells ($n = [Y]$ independent biological experiments).

Wound Healing Assay

About 5.0×10^3 cells were seeded individually in two labelled 8-chamber slides (Thermo Fisher Scientific 04231481, USA) with 200 μ L of media and incubated for 24 h at 37 °C and 5% CO₂. After forming a confluent monolayer, cells were treated with 5-Aza, 5-FU, or both to a final concentration of 5 μ M. The monolayer of the HCT116 and HEK293 cells was wounded using a 10- μ L tip. The media in all chambers of both slides were discarded, and the cells were washed with 200 μ L PBS, observed under an inverted microscope, and captured at 0 h, 3 h, 6 h, and 9 h after wounding.

The wound-healing experiment was independently performed 3 times. For each condition, 3 independent microscopic fields/wounds were quantified per experiment.

Colony Formation Assay

About 5.0×10^3 cells of each cell line were seeded in a 24-well plate with 250 μ L of 0.2% FBS media and incubated for 24 h at 37 °C and 5% CO₂. After 24 h, cells were treated with either 5-Aza, 5-FU, or both to a final concentration of 5 μ M. Later (24 h), the media was

discarded, and the cells were fixed by adding 150 μ L of absolute cold methanol (603-001-00-X, Honeywell, USA) and incubated at room temperature for 15 min. Methanol was removed, and cells were washed with 250 μ L of 1x PBS. Then, 250 μ L of 0.5% Crystal Violet diluted in 20% methanol was added to cells before the plate was placed on a rocker (SSL4, Stuart Equipment, UK) and incubated at room temperature for 15 min. The wells were washed with tap water three times before the plate was photographed using 4x and 10x magnification. The stain absorbance at 570 nm of both cell lines was also measured using the microplate reader.

The colony-formation assay was independently repeated 3 times, with 3 technical replicate wells per treatment condition in each experiment.

Whole Exome Sequencing (WES)

The HCT116 and HEK293 cells were treated with either 5-FU, 5-Aza, or both for 24 h and then harvested by trypsinization. DNA was extracted for all cells using a DNA extraction kit (ZYMO Research D3024, USA). The extracted DNA was stored in a -20° C freezer for further use. The purity and concentration of the extracted DNA samples were analyzed using a spectrophotometer (NANODROP 2000c, Thermo Scientific, USA) to meet the WES DNA quality criteria (a ratio of ~1.8 at 260/280 and a concentration of ≥ 12.5 ng/ μ L).

Based on the quality and concentration of DNA, only four samples were selected for WES: HCT116/5-FU, HCT116/0.1% DMSO, HEK293/5-FU, and HEK293/0.1% DMSO. An adenine base was added at the 3' end of each fragment, and LM-PCR was carried out for amplification of DNA fragments. Enrichment was performed by hybridizing the DNA fragments, and rolling circle amplification (RCA) of the DNA fragments was carried out to produce DNA Nanoballs (DNBs). The qualified captured library was loaded into the BGISEQ platforms, and the sequencing was performed. Raw data were obtained and stored in FASTQ format. The raw data generated were analyzed by performing bioinformatics analysis, data clean-up, mapping and marking duplicates, local realignment around InDels, Base Quality Score Recalibration (BQSR), variant calling, and variant filtering.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software,

USA). Data are presented as mean $\pm$ [SD/SEM; SELECT THE ACTUAL ONE] from [n] independent biological experiments unless otherwise indicated. Technical replicates within an experiment were averaged before inferential statistical analysis. For comparisons involving more than two treatment groups, [one-way ANOVA followed by an appropriate multiple-comparison procedure] was used; comparisons involving treatment and time were analyzed using [two-way ANOVA, if appropriate]. Two-group comparisons were performed using a two-sided unpaired t-test only where appropriate. Statistical significance was defined as $P < 0.05$.

RESULTS

MTT assay

Because DMSO has a documented cytotoxic effect, in the present study, HCT116 and HEK293 were exposed to 4 different concentrations of the solvent: 0.1%, 0.2%, 0.4%, and 0.8%. MTT assay was performed 24 h after DMSO treatment (Figure 1). Results generally showed that the highest concentration of DMSO (0.8%) yielded the most reduction in cell viability in both cell lines. The cell viability percentages for HEK293 cells were 74%, 58.2%, 31.5%, and 37.46% for 0.1%, 0.2%, 0.4%, and 0.8%, respectively. In contrast, the HCT116 cells' viability percentages were 90%, 64.3%, 34.6%, and 30% for 0.1%, 0.2%, 0.4%, and 0.8%, respectively. The treatment showed that the reduction of cell viability was concentration-dependent.

Based on these results, we decided to use DMSO in a final concentration of 0.1% in the subsequent analyses.

Challenging the two cell lines with 5-Aza and -FU for 24 h resulted in non-significant differences ($p=0.2060$) in the cell viability of the two cell lines, where the percent of cell viability was 1.69% and 2.93% for HCT116 and for HEK293, respectively (Figure 2). A significant reduction in the cell viability ($p>0.0001$) was observed in 5-FU (5 μ M), where this treatment yielded 39.61% in HCT116 and 65.28% in HEK293. Moreover, dual hit with both drugs has resulted in a significant difference ($p=0.0002$) in the cell viability, where this treatment has resulted in an 18.58% and 8.33% cell viability in HCT116 and HEK293, respectively.

When both cell lines were treated with the drugs for 48 h, 5-Aza (5 μ M) showed no significant difference between HCT116 (1.24%) and HEK293 (2.60%) ($p=0.1720$) in terms of the reduction in cell viability (Figure 3). While treating the cells with 5-FU (5 μ M) has resulted in a significant variation ($p>0.0001$) between the two cell lines, where percents of cell viability were 36.37% and 2.98% for HCT166 and HEK293, respectively. Meanwhile, treating the cells with both drugs revealed a non-significant difference ($p>0.7978$) between the cell lines, where the percentages of cell viability were 1.11% and 1.33% for HCT116 and HEK293, respectively.

For HEK293 and HCT116 cells at 72 h (Figure 4), data revealed that treating HCT116 with 5-Aza (5 μ M)

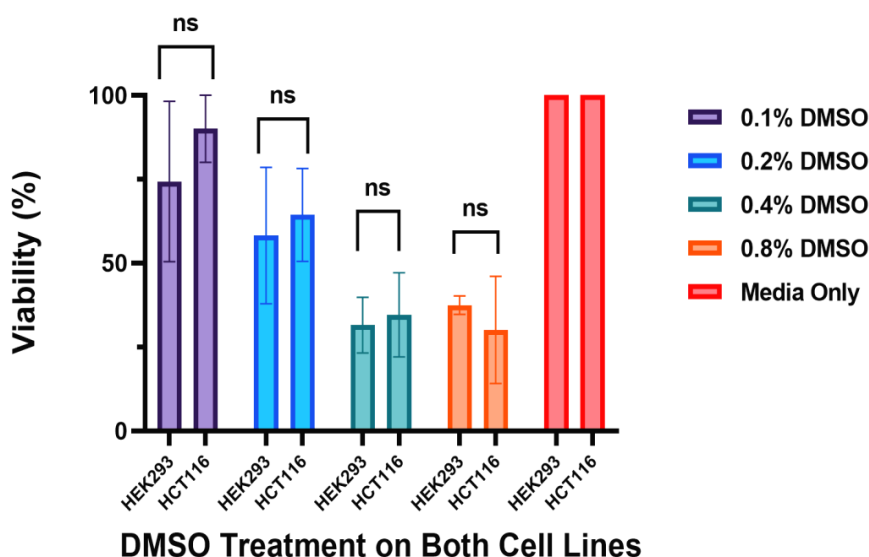


Figure 1: The cytotoxicity assay using MTT in both cell lines HEK293 and HCT116. Cells were treated with increasing concentrations of DMSO: 0.1%, 0.2%, 0.4%, 0.8%, and media only. The same treatment in both cell lines exhibited a non-significant difference ($p>0.005$). P-value is considered significant at < 0.5 as (*, **, ***).

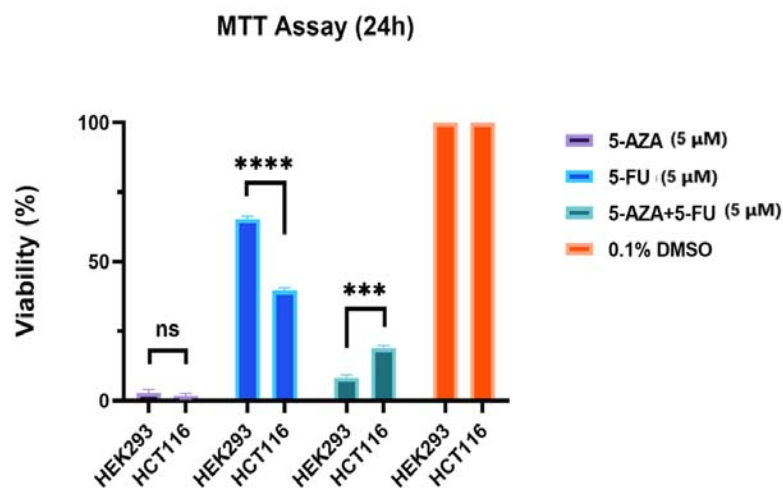


Figure 2: A histogram shows a comparison of both HEK293 and HCT116 cell viability percentages upon exposure to either 5-Aza (5 μM), 5-FU (5 μM), or 5-Aza +5-FU (5 μM).

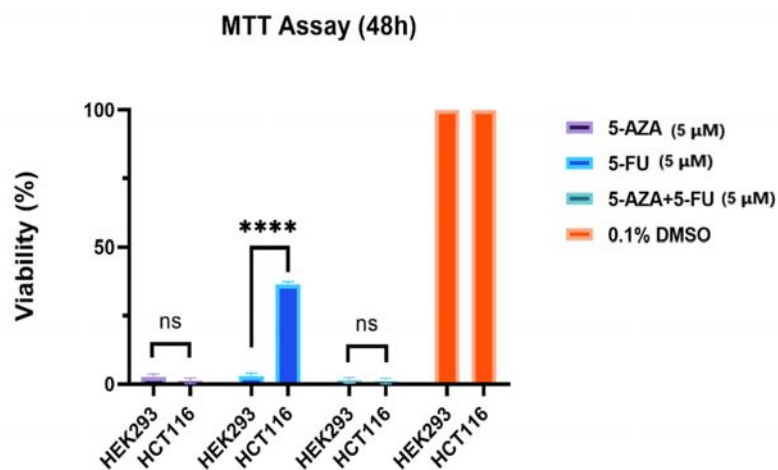


Figure 3: A histogram shows a comparison of both HEK293 and HCT116 cell viability percentages upon exposure to either 5-Aza (5 μM), 5-FU (5 μM), or 5-Aza +5-FU (5 μM).

has resulted in a significant reduction ($p > 0.0001$) in the cell viability compared to the normal cells; HEK293, with a cell viability percentage of 22.42% for HCT116 cells and 51.21% for HEK293 cells. The same profile was observed when cells were treated with 5-FU (5 μM), where the HCT116 cells were more sensitive to the drug than the normal cells ($p > 0.0001$). In HCT116, the cell viability percentage was 16.54% compared to 53.65% for HEK293. When cells were treated with a combination of the two drugs (neoadjuvant therapy), HCT116 cells were significantly affected ($p > 0.0001$) compared to HEK293. The cell viability was 33.45% in HCT116 and 4.87% in HEK293.

Wound Healing Assay

HCT116 colon cancer cells were treated with either 5-FU, 5-Aza, or a combination of the two drugs to

assess the inhibitory effect of these drugs on the migration of HCT116 cells. Wound healing assay was performed on the drug-treated and DMSO-treated cells, and readings were recorded after 0 h and 9 h. Data revealed that after 9 h of treating the cells with the drugs/drug combination. After 9 h, treatment-dependent differences in wound closure were observed relative to the DMSO control. Reduced wound closure was observed following exposure to 5-Aza, 5-FU, and the combined treatment. Because these treatments also affected cellular viability, the scratch-assay findings cannot distinguish a migration-specific effect from reduced proliferation and/or treatment-associated cytotoxicity (Figure 5).

We further analyzed the effect of the same drugs/combination on the migration of HEK293 cells (Figure 6).

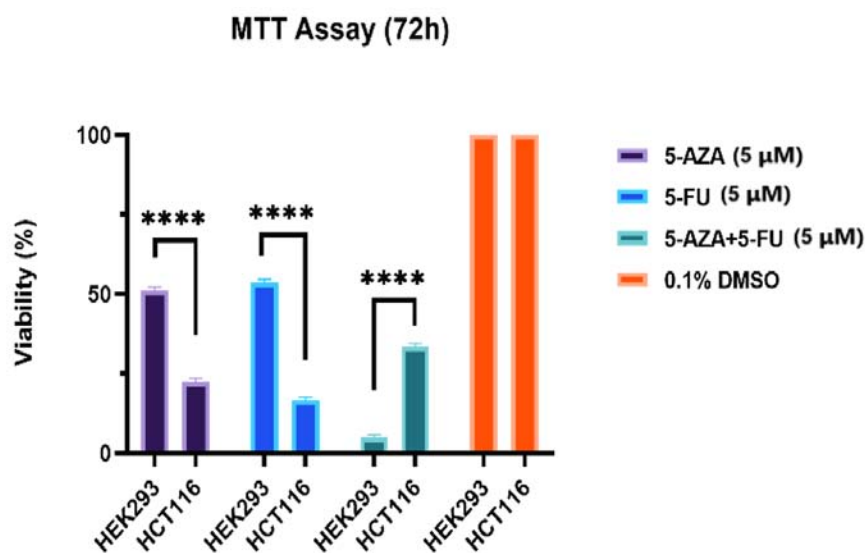


Figure 4: A histogram shows a comparison of both HEK293 and HCT116 cell viability percentages upon exposure to either 5-Aza (5 μ M), 5-FU (5 μ M), or 5-Aza +5-FU (5 μ M).

Colony Formation Assay

Further, we assessed the ability of the treated and untreated cells to form colonies. The results indicated that the colony-forming ability was reduced in either HCT116 or HEK293 cells after being treated with 5-FU and 5-Aza (Figure 7A-H). Crystal Violet staining was used to assess colony-associated cellular biomass following treatment. Relative absorbance at 570 nm was reduced in several treatment groups compared with the DMSO control, consistent with reduced colony-forming capacity and/or retained cellular biomass. These measurements do not distinguish among potential mechanisms responsible for the reduction (Figure 7I).

HCT116 cells showed a numerical reduction in absorbance compared with the DMSO control; however, this difference did not reach statistical significance ($P = 0.0541$) in the absorbance compared to 0.1% DMSO treatment, and the rates were 42.89% and 100% for 5-FU and 0.1% DMSO, respectively. Furthermore, HCT116 was also treated with the dual drugs, and the colony-forming assay results indicated that the absorbance rate was significantly reduced ($p=0.0049$) compared to treating the same cells with 0.1% DMSO. Rates were 28.3% and 100% for 5-FU+5-Aza and 0.1% DMSO, respectively.

Conversely, in HEK293 cells, an overall reduction in absorbance was observed in all treatments compared to the 0.1% DMSO. Rates were 1.04% and 100% for 5-Aza and 0.1% DMSO, respectively ($p = 0.0065$), 38.87% and 100% for 5-FU and 0.1% DMSO,

respectively ($p=0.0414$), and 25.79% and 100% for 5-FU+5-Aza and 0.1% DMSO, respectively ($p=0.0024$).

Whole Exome Sequencing

In the present study, we treated HCT116 and HEK293 cells with either 5-Aza, 5-FU, or a combination of the two drugs. We analyzed the whole exome to understand further the molecular mechanism by which these drugs affect colon cancer cells. We checked the quality of DNA before performing WES. Only four samples were selected to perform WES: HCT116 cells treated with 5-FU, HCT116 cells treated with 0.1% DMSO, HEK293 cells treated with 5-FU, and HEK293 cells treated with 0.1% DMSO.

After removing the low-quality reads of whole-exome sequencing and processing the raw data obtained to get clean reads, high-quality sequencing quality results of the four selected samples were obtained. 80.20% of the desired data was captured in target regions, which the BGISEQ software addresses as capture specificity. The resulting averaged GC content among the 4 samples is 50.68%, while the average rate of clean data obtained is 92.41%.

After sequencing, 17,349 single-nucleotide polymorphisms (SNPs) were identified in the four selected samples. 54.76% of these SNPs were already present in the Single Nucleotide Polymorphism Database (dbSNPs), while 46.73% were in the 1000 Genomes Project database. Interestingly, 7,845 SNPs were novel (Table 1).

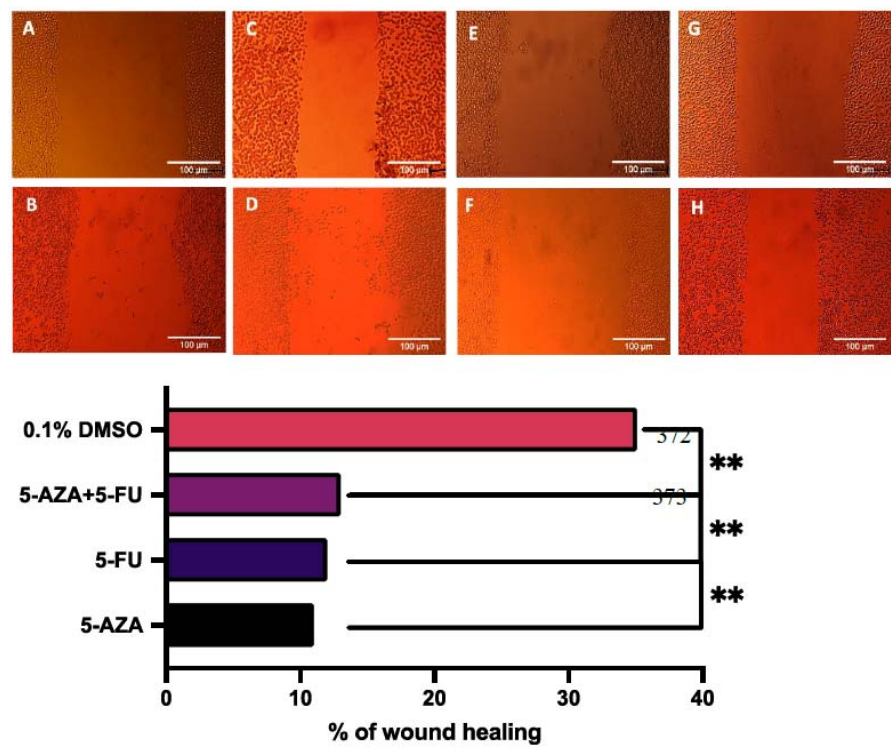


Figure 5: HCT116 cells treated with: **A:** 5-Aza -0h, **B:** 5-Aza -9h, **C:** 5-FU-0h, **D:** 5-FU-9h, **E:** 5-Aza +5-FU-0h, **F:** 5-Aza +5-FU-9h, **G:** 0.1%DMSO-0h, and **H:** 0.1% DMSO-9h.

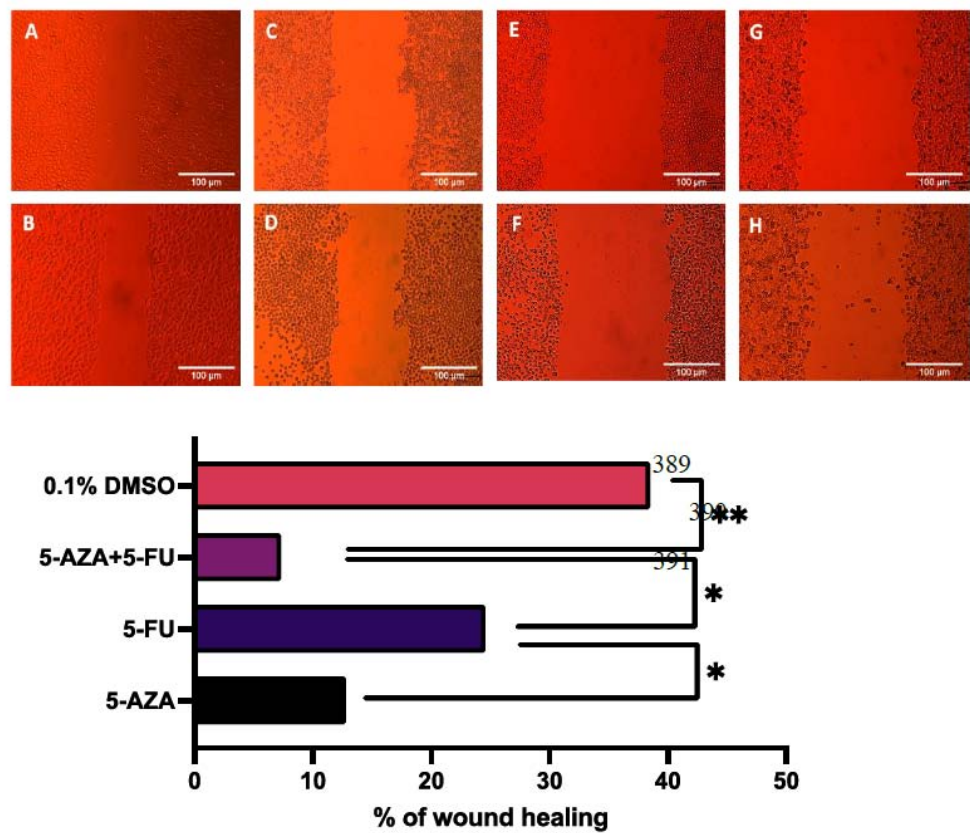


Figure 6: HEK293 cells treated with: **A:** 5-Aza -0h, **B:** 5-Aza -9h, **C:** 5-FU-0h, **D:** 5-FU-9h, **E:** 5-Aza +5-FU-0h, **F:** 5-Aza +5-FU-9h, **G:** 0.1% DMSO-0h, and **H:** 0.1% DMSO-9h.

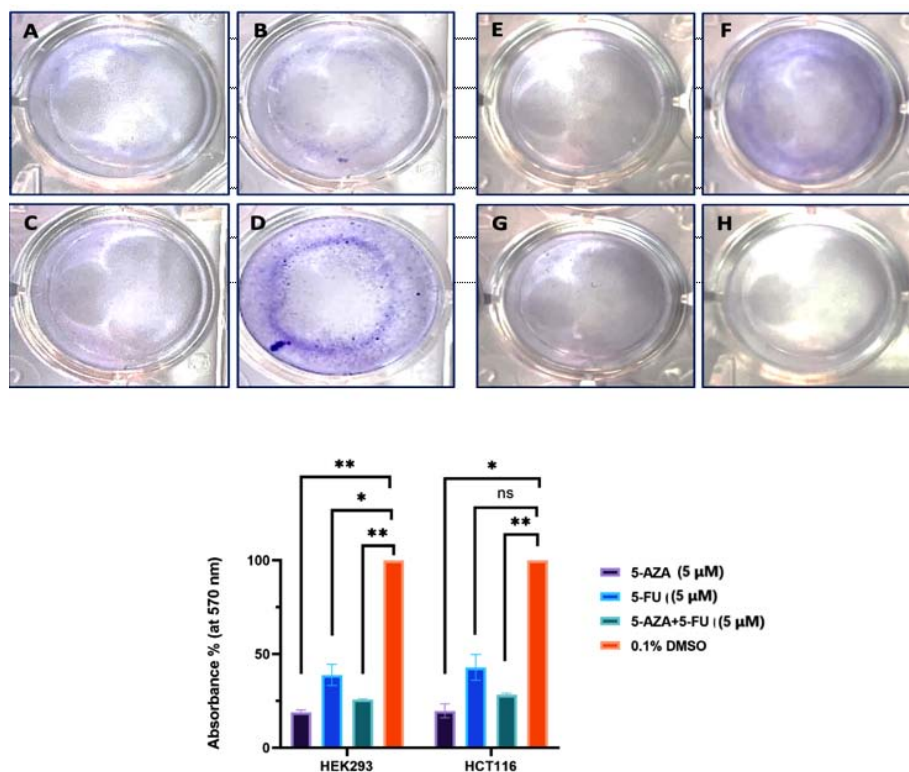


Figure 7: Colony forming assay for both HCT116 and HEK293 cells treated with: **A:** HCT116/5-Aza, **B:** HCT116/5-FU, **C:** HCT116/5-Aza+5-FU, **D:** HCT116/0.1 % DMSO, **E:** HEK293/5-Aza, **F:** HEK293/5-FU, **G:** HEK293/5-Aza+5-FU, **H:** HEK293/0.1%DMSO, and **I:** the absorbance of bromophenol blue in treated and untreated cells.

Table 1: Summary Statistics for Identified Single Nucleotide Polymorphisms (SNPs)

Samples	Total SNPs	Fraction of SNPs in dbSNPs%	Fraction of SNPs in 1000 G%	Novel
HCT116/5-FU	4,213	16.43	4.39	3,521
HCT116/0.1% DMSO	249,448	22.85	11.63	192,448
HEK293/5-FU	444	11.94	2.25	391
HEK293/0.1% DMSO	545	12.29	2.94	478

Among the known SNP types, 5,161 SNPs of the 4 samples were synonymous, 3,182 were missense, 1 was stop-loss, 60 were stop-gain, 3 were start-loss, and 22 were splice site (Figure 8A). Furthermore, among the four selected samples, there were 12,468 InDels; 0.27% were present in dbSNPs and 0.06% in the 1000 Genomes Project database. The novel InDels were 12,434 (Figure 8B). A summary of the statistics for the identified InDels is represented in Figure 8C.

DISCUSSION

The present study analyzed the effect of 5-FU and 5-Aza on HCT116 cells. MTT assay indicated that 5-Aza, as a DNA demethylation agent, has significantly reduced the cell viability of HCT116 and HEK293 cells compared to DMSO (0.1%) treatment. However, the

malignant cells were more sensitive to the drug. This might be attributed to the upregulation of several growth restriction genes such as p16INK4a, p14ARF, and p15INK4b [21] caused by 5-Aza. Meanwhile, 5-FU, the first line of CRC treatment [22], also affected the overall viability in both cells, with HCT116 being more sensitive to this drug. It is well documented that the efficiency of 5-FU as a potent anticancer agent is impaired by its resistance in different cancer types [23, 24]. To alleviate this resistance, combining 5-FU with other chemotherapeutic drugs could help. In the present study, we treated CRC cells with 5-FU and 5-Aza. Our results indicated that although the combination had a severe effect on the viability of cells, the monotherapy of 5-Aza was more effective in reducing the cell viability in HCT116 cells. This could be explained in light of the final concentrations used for

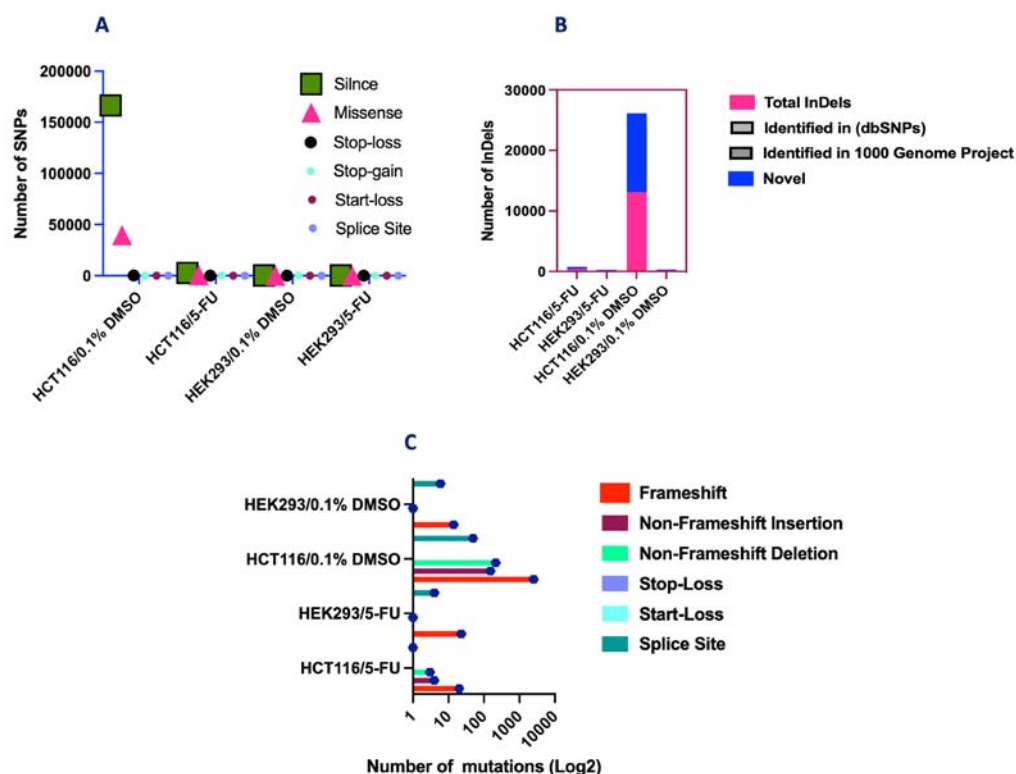


Figure 8: The identified SNPs upon treating cells with 5-FU. **A:** Functional categories for the coding single-nucleotide polymorphisms (SNPs), **B:** The total InDels and those recorded in the SNPs database and identified earlier in the 1000 Genomes Project. The novel SNPs discovered in the present study were also shown. **C:** Summary of the types of SNPs identified in the present study.

both drugs, 2.5 μ M from each drug compared to the 5 μ M of 5-Aza. The effect of these two drugs has not been investigated in a single study [25, 26] *et al.* have indicated that methylation status might affect the sensitivity of CRC cells to 5-Fu [26]; however, other studies have suggested that the effect of 5-Aza was weaker than that of 5-Fu on HCT116 cells [25].

We investigated cellular migration upon treatments with 5-Aza and 5-FU on HCT116 and HEK293 cells. Data indicated that for HCT116 cells, all treatments significantly inhibited cellular migration compared to the DMSO treatment. 5-FU has been extensively used as an antineoplastic agent [27] by exerting its function as an anti-metabolite that inhibits the growth of cancer cells and the DNA repair mechanism. On the other hand, 5-Aza, the demethylating agent that caused a reduction in genomic levels of 5-methylcytosine [28], can also inhibit cellular migration via its role in modifying pyrimidine and impair normal cell division.

The treatments for normal cells, HEK293 could inhibit the wound healing process compared to the DMSO treatment; the dual-drug treatment was more potent in impairing cellular migration.

Meanwhile, we also assessed the ability of treated cells to form colonies as an indicator of their viability. Data showed that the absorbance of bromophenol blue by the treated and untreated cells differed among treatments. Compared to the DMSO treatment, a significant reduction in the retention ability was obtained in HCT116 cells treated with either the sole drugs or the drug combination. The same profile was observed in HEK293 cells. The reduction in colony-associated staining following treatment is consistent with impaired long-term clonogenic survival. However, this assay does not identify the mechanism underlying the reduction, and apoptosis cannot be inferred in the absence of apoptosis-specific measurements such as Annexin V/PI staining, caspase activation, or detection of apoptosis-related proteins. However, it must be combined with a neoadjuvant drug to minimize its resistance by colon cancer cells, such as allicin [29] and curcumin [30].

Furthermore, 5-Aza was found to induce anoikis in different types of cancer, including breast cancer, at a concentration resembles that what has been used in the present study (8.014 μ M), and this inhibits the formation of tumor sphere, migration, as well as inhibits

the activity of metalloproteinases-9 (MMP-9) [31]. Furthermore, 5-Aza blocks the DNMT enzyme activity, which is responsible for methylating the DNA, by incorporating into the DNA [32]. As a result, blocking DNMT will be more evident in cancer cells than in normal cells because it is associated with cancer-related factors [33].

For a deeper look at the action of 5-FU on HCT116 and HEK293 cells, we further analyzed the whole exome. Data indicated that HCT116 treated with DMSO (0.1%) showed 249,448 SNPs, while the 5-FU treatment reduced the number of SNPs to 4,213. Typically, malignant cells encompass a significant number of mutations. The same was observed in HEK293 cells, where the native number of SNPs in DMSO treatment was 545 and reduced to 444 with the 5-FU treatment. This might indicate the DNA-damaging effect of 5-FU that has resulted in fewer mutations. Moreover, the number of SNPs detected in HCT116 cells treated with 5-FU (2,830) was identified as synonyms, which can be interpreted as silent mutations that will not cause a change in amino acid production. Moreover, compared to the DMSO treatment, the 5-FU treatment significantly reduced the number of frameshifts, non-frameshift deletions, stop-loss, start-loss, and splice site mutations.

It has been indicated that 5-FU treatment in HCT116 cells could reduce the number of stop-loss and stop-gain SNPs. These mutations can lead to severe cellular problems. For instance, sudden truncation of the protein translation process caused by the stop-gain mutation can prevent the production of many proteins that might contain tumor suppressor genes [34]. Also, start-loss can prevent the synthesis of proteins that might also include tumor suppressor genes to prevent cancer initiation. In addition, 5-FU treatment caused a significant reduction in the splice site SNPs, the mutations that occur at the splice site region between an intron and an exon. These mutations can disrupt the mRNA's RNA splicing, resulting in an undesirable change in the protein-coding sequence [35].

The results obtained in the present study indicated that 5-Aza could sensitize HCT116 cells to 5-FU, and this combination therapy represents a potential approach to combat CRC.

CONCLUSION

Resistance to 5-FU remains an important limitation of colorectal cancer therapy, providing a rationale for

investigating agents that may modify cellular responses to fluoropyrimidines. In the present *in vitro* study, combined exposure to 5-Aza and 5-FU altered HCT116 viability, wound closure, and colony-forming capacity. Because the study did not employ a multidose combination matrix or formal drug-interaction modeling, these findings should not be interpreted as demonstrating pharmacological synergy. Moreover, the responses observed in HEK293 cells indicate that cancer-cell selectivity cannot be established from the present model. Further studies using dose-response matrices, validated non-transformed colorectal epithelial controls, mechanistic assays, and formal synergy analyses are required to determine whether 5-Aza can meaningfully enhance the therapeutic response to 5-FU.

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AUTHOR CONTRIBUTIONS

HS: conceptualized the idea and approved the first draft, TA: conducted the experimental work, SA-G: participated in writing the draft and practical work. HB and AE: revision, editing and submission.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE:

Not applicable.

DATA AVAILABILITY CONSENT

All data generated are included in this manuscript.

CONSENT FOR PUBLICATION

All authors have read and approved submission.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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