RESEARCH ARTICLE

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Sodium Butyrate Activity in Colorectal Cancer under Hypoxia: A Potential Role for Carbonic Anhydrase IX

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Abstract

Background/Aim: Hypoxia poses a significant challenge to cancer therapy. The impact of hypoxia on butyrate, a gut metabolite that has anti-colorectal-cancer properties, is unclear. Thus, the aim of this study was to determine the activity of sodium butyrate (NaB) under hypoxic conditions. Furthermore, this study was the first to investigate the potential of carbonic anhydrase IX (CA IX), a hypoxia-regulated gene, as a target for NaB. **Materials and Methods:** The *Caco-2* and *HT-29* colorectal cancer cell lines were exposed to 0, 5, 10, 15, and 20 mM NaB under hypoxic and normoxic conditions for 24 and 48 hours. We assessed cell viability and clonogenicity rates using an MTT assay and a colony-forming assay, respectively. RT-PCR was used to measure the effect of NaB on CA IX expression level. **Results:** After 24 hours of treatment with NaB, the percentage of viable *HT-29* cells decreased under normoxic conditions, while it remained unchanged under hypoxic conditions. After 48 hours, the percentage of viable *HT-29* cells decreased under both oxygen conditions. The viability of normoxic and hypoxic *Caco-2* cells was only reduced after 48 hours of NaB application. Interestingly, NaB significantly reduced the number of *HT-29* and *Caco-2* colonies in normoxic and hypoxic conditions. NaB significantly decreased CA IX mRNA expression level in *HT-29* hypoxic cells after 48 hours ($p < 0.0001$), but not after 24 hours. **Conclusion:** At the beginning, hypoxia caused an alteration in the anti-colorectal-cancer activity of NaB, which then returned to normal. Our data revealed the novel finding that NaB downregulates CA IX, suggesting a potential therapeutic strategy for colorectal cancer that targets tumor metabolism and pH regulation.

Keywords: Sodium butyrate- Normoxia- Hypoxia- Carbonic anhydrase IX- Colorectal cancer

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer in terms of incidence (1.9 million) and the second leading cause of cancer-related mortality (0.9 million) worldwide in 2022. These numbers are expected to double by 2045 [1]. Today, strong evidence links gut microbiota types and their metabolites to CRC development and prevention [2]. CRC causative microorganisms can promote tumorigenesis by inducing inflammation, DNA damage, or cell growth signaling pathways activation [3]. Protective bacteria enhance the mucosal immune response and produce antitumor metabolites [4, 5]. Butyrate, a short-chain fatty acid (SCFA) produced by Firmicutes bacteria, appears to be one of principal gut microbial metabolites that protects against CRC [6]. Patients with CRC showed reduced levels of butyrate-producing bacteria compared to healthy individuals [7, 8]. In vitro studies demonstrated that NaB has a strong anti-proliferative effect against *HT-29* and *Caco-2* human colorectal cancer cells [9].

Hypoxia, a hallmark of tumor microenvironment (TME), remains a key challenge for CRC therapy. The effect of hypoxia on butyrate's activity in CRC is limited and requires further investigation. Interestingly, several studies showed that NaB disrupts the hypoxia pathway leading to cancer cells death. In fact, NaB downregulates the expression of the hypoxia- inducible factor-1α (HIF-1α) and its downstream effectors in human colorectal cancer HCT-116 cells under both normoxic and hypoxic conditions [10]. Furthermore, two previous studies have shown that NaB downregulates HIF-1α and the vascular endothelial growth factor VEGF, mediator of angiogenesis in *HT-29* and HT1080 human fibrosarcoma cells [11, 12]. In CT26 murine colorectal cancer cells, under hypoxia, NaB inhibited the AKT/mTOR/HIF-1α pathway and suppressed aerobic glycolysis [13].

In addition to HIF-1α and VEGF, carbonic anhydrase IX (CA IX) is another major hypoxia-regulated gene that is required for cell survival in an acidic TME. Solid tumors with high levels of CA IX are more likely to progress and metastasize [14]. CA IX regulates the pH of hypoxic cancer cells by exporting lactate derived from glycolysis and increasing proton generation in the extracellular

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compartment. However, no studies have investigated the effect of NaB on CA IX expression level in CRC. Thus, this study aimed to determine how hypoxia affects the anti-cancer activity of NaB and subsequently the CA IX expression level in CRC providing insight into a potential therapeutic mechanism.

Materials and Methods

Cell Culture

Caco-2 and *HT-29* human colorectal cancer cell lines were used in this study. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO™) supplemented with 10% fetal bovine serum (FBS; GIBCO™) and 1% penicillin–streptomycin (GIBCO™), and maintained at 37°C in a humidified incubator with 5% CO_2. Cells were routinely subcultured at approximately 80% confluence and used for experiments between passages 15 and 30 to ensure phenotypic consistency. All experiments were conducted using independently cultured cell populations.

NaB and Hypoxia Treatment

Sodium butyrate (NaB; 98%, Sigma-Aldrich, St. Louis, MO, USA) was prepared in culture medium and applied at final concentrations of 0, 5, 10, 15, and 20 mM. Cells were incubated under normoxic conditions (95% air, 5% CO_2) or hypoxic conditions for 24 or 48 hours. Hypoxia was induced using sealed hypoxic jars in combination with oxygen-depleting sachets, designed to reduce oxygen levels to approximately <1–2% O_2, a range commonly used to model tumor-relevant hypoxic conditions in vitro. Functional induction of hypoxia was inferred through differential cellular responses and modulation of the hypoxia-responsive gene carbonic anhydrase IX (CA IX). Untreated cells (0 mM NaB) served as controls in all experiments.

Cell Viability Assay

Cell viability and metabolic activity were assessed using the MTT assay (Ab211091, Abcam). *HT-29* cells (10,000 cells/well) and *Caco-2* cells (8,000 cells/well) were seeded in 96-well plates and allowed to attach for 48 hours prior to treatment. Cells were then treated with NaB and incubated under normoxic or hypoxic conditions for 24 or 48 hours, as specified. Each condition was assessed in technical quadruplicates. Absorbance was measured at 590 nm, and viability was calculated relative to untreated controls after blank correction. As the MTT assay reflects mitochondrial metabolic activity, changes in viability may indicate altered proliferation and/or metabolic suppression rather than direct cytotoxicity.

Colony Forming Assay

HT-29 and *Caco-2* cell lines were used in this assay and samples were run in duplicates for each condition. After inducing hypoxic/normoxic effects and treatment, cells were seeded in a 6-well plate in a very low density of 150 cells per well for *HT-29* and 120 cell per well for *Caco-2*. Cells were then incubated for 7 days to allow colony formation. After incubation, cells were washed with phosphate buffered saline (PBS) then fixed with methanol at room temperature to immobilize colonies. After fixation, 0.5% crystal violet was used for staining and cells were washed distilled water and air-dried at room temperature for 24 hours. Using a tissue culture microscope, plates were pictured, and colonies were counted manually.

Quantitative Reverse Transcription PCR (qRT-PCR)

CA IX expression analysis was restricted to the *HT-29* cell line due to its documented basal and hypoxia-inducible expression in this cell line. Publicly available proteomic datasets further indicate detectable CA IX expression in *HT-29* cells, while there is no enough information about its expression in *Caco-2* cells. So, Total RNA was extracted from *HT-29* samples treated with 0 and 5 mM NaB under normoxic and hypoxic conditions. miRNeasy Mini or Micro kit from Qiagen was used for RNA isolation quantification. Quality assessment of RNA was carried out using the NanoDrop spectrophotometer (ThermoFisher). After detection of RNA concentration, 2 µg of total RNA was reverse transcribed into cDNA with random primers, using the using SOLIScript® RT cDNA synthesis KIT. The amplification reaction was performed using the PowerUp SYBR Green Master Mix Kit from ThermoFisher Scientific. β-actin gene was used as an internal control. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of target genes. All the samples were run in triplicates. CA IX primers gene was purchased from Microgen and sequence is as follows: Forward: TTTGCCAGAGTTGACGAGGC, Reverse: GCTCATAGGCACTGTTTTCTTCC. Some studies have used β-actin as a housekeeping gene under hypoxia [15].

Statistical Analysis

GraphPad Prism 10 software was used for statistical analysis. Data are presented as mean ± standard deviation (SD). For in vitro assays, a two-way analysis of variance (ANOVA) was applied to assess the effects of sodium butyrate concentration and oxygen conditions. A p-value < 0.05 was considered statistically significant.

Results

Effects of NaB on the cell viability of colorectal cancer cells in normoxia and hypoxia

Human colorectal cancer cells *HT-29* were exposed to a concentration gradient of NaB for 24 and 48 hours. As expected, under normoxic conditions, NaB significantly decreased the percentage of *HT-29* viable cells. The percentage of viable cells decreased to 43%, 40% and 17% for 5, 15 and 20 mM NaB, respectively after 24 hours. With an extended incubation for 48 hours, cell viability of 64%, 26%, and 24% was observed across the NaB concentration gradient. However, under hypoxic conditions, the percentage of viable cells did not change after 24 hours ($p > 0.05$), but decreased to 77% and 74% after 48 hours for 5 mM and 20 mM, respectively (Figure 1A-B).

In *Caco-2* cells under normoxic or hypoxic conditions, NaB did not reduce cell viability after 24 hours, but

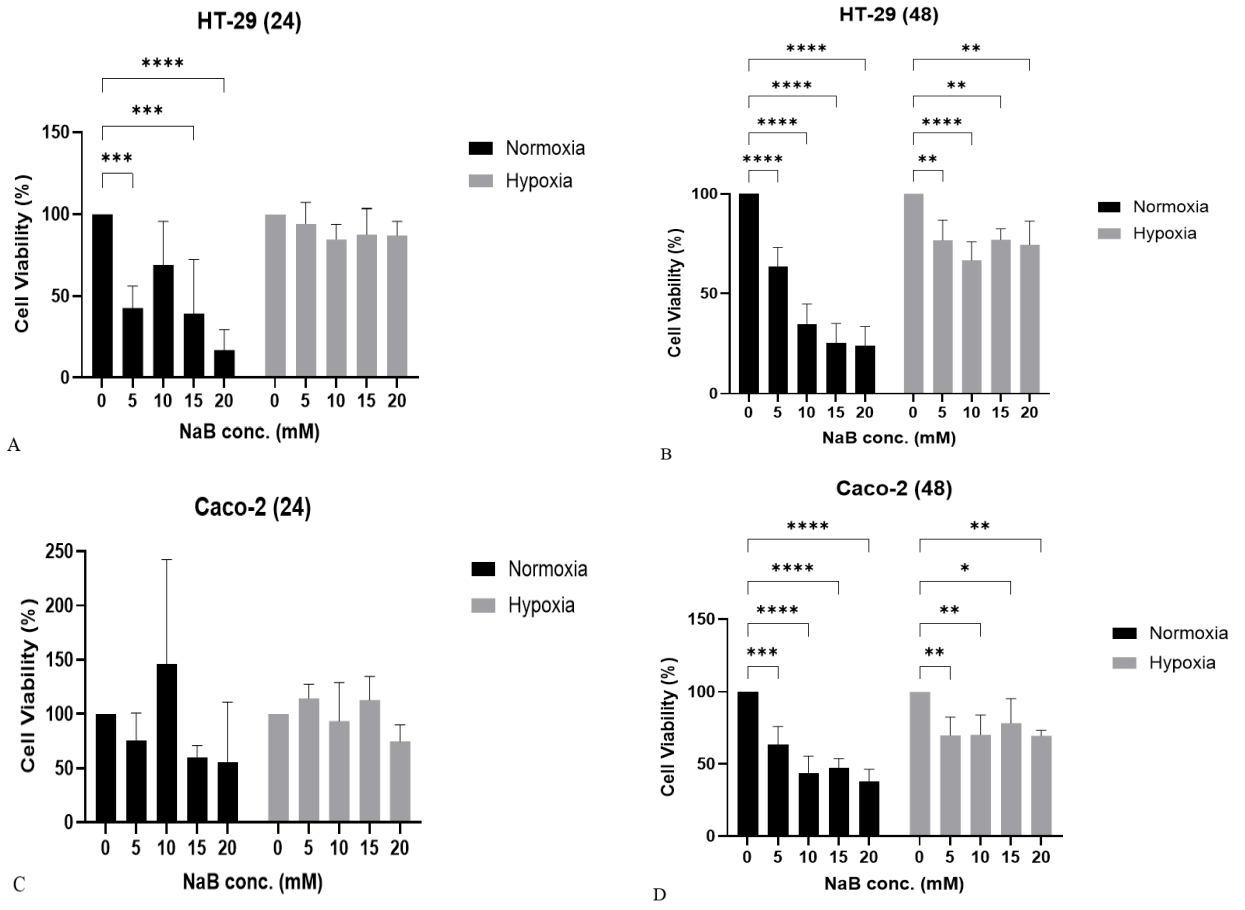

Figure 1. Percentage of Cell Viability of *HT-29* and *Caco-2* in different NaB and Oxygen Conditions. Cells after 24 and 48 hours of NaB Exposure under Normoxic and Hypoxic Conditions. *HT-29* (A-B) and *Caco-2* cells (C-D). Cells were exposed to 0, 5, 10, 15 and 20 mM of Sodium Butyrate (NaB). 0 mM concentration is considered as the control. Each condition was assessed in quadruplicates. A two-way analysis of variance (ANOVA) was applied for comparison. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

it did reduce viability after 48 hours. The viability of normoxic cells was 64% with 5 mM NaB, 44% with 10 mM NaB, and 38% with 20 mM NaB. For most NaB concentrations, cell viability of hypoxic cells decreased to 70% (Figures 1C–D). A similar pattern of *Caco-2* cell viability in normoxic conditions treated with NaB was observed in a previous study [16]. Compared to *HT-29* cells, *Caco-2* cells are slow-growing, with a doubling time exceeding 24 hours. This explains the absence of an inhibitory effect of NaB on *Caco-2* cells at 24 hours. Notably, the inhibitory effect of NaB was less pronounced under hypoxic conditions than under normoxic conditions, particularly at higher concentrations in both cell lines ($p < 0.05$).

Effects of NaB on clonogenic ability of colorectal cancer cells in normoxia and hypoxia

A clonogenic assay was performed to test the effect of a 24-hour pre-treated cells with 5 mM NaB on the clonogenicity of CRC cells. After one week of incubation, the number of colonies formed by *HT-29* cells pretreated with NaB for 24 hours under normoxia decreased from 72.5 to 61 ($p = 0.0066$). These results suggest that 24 hours of pretreatment with NaB reduces the proliferative ability of a large group of cells under normoxic conditions. Similarly, cells pretreated with NaB for 24 hours under hypoxic conditions exhibited a greater decrease in colony formation than under normoxic conditions, with a reduction from 98.5 to 68.5 colonies ($p = 0.0002$). Thus, a significant proportion of cells lost their proliferative ability despite surviving in hypoxia for 24 hours (Figure 2A). *Caco-2* cells that were pretreated with NaB for 24 hours under both normoxic and hypoxic conditions showed a significant decrease in colony number compared to the control group (Figure 2B). This indicates that the ability of *Caco-2* cells to proliferate was reduced despite their survival in these conditions.

Effects of NaB on Carbonic Anhydrase IX mRNA expression level in normoxia and hypoxia

We measured the effect of NaB on CA IX mRNA expression level, a well know hypoxia hallmark in tumors [17]. The measurement was only performed in the *HT-29* cell line, as CA IX expression level is known to be detected under normoxia and highly induced under hypoxia in this cell line [18]. After 24 hours, treatment with 5 mM NaB resulted in substantial upregulation of CA IX gene expression under normoxic conditions, with a fold change of 33.94 compared to the control ($p < 0.0001$). However, there was no change in CA IX expression level after 24

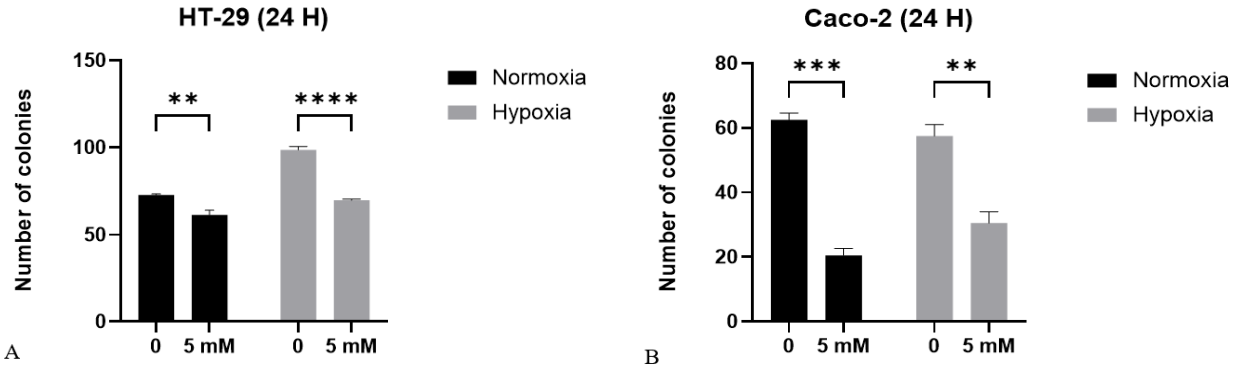

Figure 2. Effects of NaB on Clonogenic Ability. This figure illustrates the number of colonies at 0 and 5 mM NaB concentrations in normoxia and hypoxia at 24 hours. Results of *HT-29* (A) and *Caco-2* cells (B) tests are presented. The control is considered 0 mM NaB. Each condition was assessed in duplicates. A two-way analysis of variance (ANOVA) was applied for comparison. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

hours of NaB treatment under hypoxic conditions (Figure 3 A). The observed upregulation of CA IX expression in normoxic cells could be explained as an adaptive response to survive under the inhibitory effect of NaB, since the basal level of CA IX expression is very low when oxygen is available. The unchanged level of CA IX expression in hypoxic cells treated with NaB might explain their normal survival rate. In contrast, there was a significant downregulation of CA IX expression level both in normoxia and hypoxia at 48 hours ($p < 0.0001$) (Figure 3 B). A decrease in the expression level of CA IX could explain the decrease in cell viability under both conditions.

Discussion

Butyrate is currently classified as a short-chain fatty acid with anti-cancer properties against colon cancer cells in vitro [19]. New evidence suggests that NaB inhibits the aerobic glycolysis pathway that cancer cells use to survive in hypoxic conditions. However, hypoxia is considered as a key factor in drug resistance in cancer therapy [20–22]. Little is known about how hypoxia affects butyrate's anti-proliferative properties in CRC.

CA IX is a key hypoxic gene that indicates how cells adapt to new metabolic changes posed by acidic hypoxic conditions [23]. Acidosis, resulting from the accumulation of metabolic by-products such as lactate, protons, and carbon dioxide in the extracellular space, enhances cancer cell invasion and metastasis. Our study is the first to examine the anti-proliferative activity of NaB in hypoxic conditions with a particular focus on the expression level of CA IX in *HT-29* cells.

As expected, the inhibitory effect of NaB was observed in normoxic HT 29 cells at 24 and 48 hours as shown in previous studies [24,25]. However, this effect was only seen at 48 hours under hypoxic conditions. The absence of the NaB effect after 24 hours of hypoxia can be explained by the normal CA IX RNA expression level that regulates pH and maintain cell survival. The NaB maintains its anti-cancer effect after 48 hours, possibly by downregulating the level of CA IX RNA expression and subsequently reducing acidic environment. Surprisingly, after 24 hours of NaB treatment, normoxic cells responded with an upregulation of CA IX. This could be a rescue mechanism since the basal level of CA IX is very low when oxygen is available [18]. These results are consistent with other

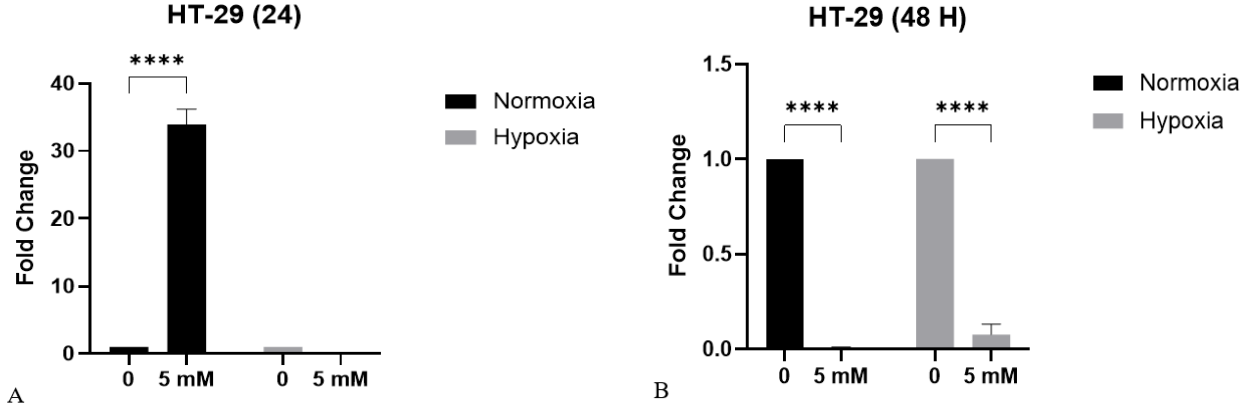

Figure 3. Effect of NaB on Carbonic Anhydrase IX mRNA Expression Level. The results of the analysis are presented, showcasing the relative gene expression changes in response to the treatment NaB 5mM, oxygen conditions, and 2 timepoints, 24 (A) and 48 hours (B). Each condition was assessed in triplicates. A two-way analysis of variance (ANOVA) was applied for comparison. ****, $p<0.0001$.

studies, which have shown that NaB inhibits HIF-1α hypoxic pathway and downstream target genes [10]. Subsequently, the results suggest that targeting CA IX via NaB could be an effective way to inhibit CRC progression [26]. NaB could be added to many other CA IX inhibitors, including small molecules such as the sulfamate inhibitor S4 and antibodies that impair tumor growth by preventing pH regulation [27]. It is worth noting that hypoxia altered the efficacy of NaB by delaying its effectiveness until 48 hours. This indicates that the hypoxic TME interacts with tumor cells by maintaining a normal carbonic anhydrase IX (CA IX) expression level to support tumor growth and resistance to NaB. In general, the effectiveness of NaB was significantly lower in hypoxic cells than in normoxic ones at higher concentrations.

It was clear that NaB influenced the clonogenicity of *HT-29* and *Caco-2* cells in all conditions after 24 hours. However, the decrease in the number of hypoxic *HT-29* colonies without a change in the viability rate indicates that hypoxia didn't affect the anti-proliferative property of NaB in the long term. Further studies are needed to decipher the molecular mechanism linking NaB to the downregulation of CA IX expression. It will be necessary to track CA IX expression at the protein level under various oxygen conditions.

This study has certain limitations. The use of a chemical hypoxia induction system, which may result in some variability in oxygen tension compared to controlled tri-gas incubators. However, such systems are widely used to model acute hypoxia in vitro and are effective in activating hypoxia-responsive cellular pathways, Additionally, the pH of the intracellular and extracellular compartments was not verified in all conditions. CA IX expression was only measured at the transcriptional, rather than the protein, level. While protein-level validation of CA IX would further strengthen these findings, the present study focused on transcriptional regulation as an initial mechanistic insight into NaB activity under different oxygen conditions. Next, it would be essential to investigate the NaB effect on CA IX expression in an in vivo pre-clinical model for colon cancer.

In conclusion, our data demonstrated that hypoxia alters NaB activity for 24 hours but not after that. This study reveals the novel finding that NaB downregulates CA IX, suggesting a potential colorectal cancer therapeutic strategy targeting tumor metabolism and pH regulation.

Author Contribution Statement

Conceptualization and design: S.M., M.H and A.S., experimentation: H.A., analysis: S.M., M.H. and H.A., work supervision: S.M. and A.S. manuscript writing: S.M. reading manuscript: all authors.

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This work is part of an approved student thesis.

Ethical statement

This study involved only established human colorectal cancer cell lines and did not include human participants, animal subjects, or clinical samples. Therefore, in accordance with the institutional policies of Beirut Arab University (BAU) and Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), ethical approval or informed consent was not required. Also, this study did not require registration in a public database, as it does not involve a clinical trial, human participants, animal experiments, systematic review, or meta-analysis.

Conflicts of Interest

All Authors declare no conflicts of interest in relation to this study.

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