Butyrate and Resveratrol synergize to inhibit colorectal cancer cell proliferation and migration by preventing the nuclear translocation of β-catenin

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Abstract Colorectal cancer (CRC) ranks as the second leading cause of cancer-related mortality worldwide. Disease progression is primarily driven by disruptions in the Wnt/β-catenin pathway, alongside alterations in gut microbiota. Accordingly, epidemiological studies support the evidence that a large portion of colorectal cancers can be prevented by modifying one's lifestyle, particularly through dietary intake of prebiotics, probiotics, postbiotics, and nutraceuticals that reduce intestinal inflammation and promote intestinal cell homeostasis and barrier integrity. In this context, targeting both microbiota and Wnt/β-catenin pathway represents a promising therapeutic approach. Here, we show that butyrate (the most abundant postbiotic produced by gut microbiota) and resveratrol (a nutraceutical counteracting IL-6-mediated inflammation) can reduce the motility and growth of CRC cells. HCT116 colorectal cancer cells were exposed to sodium butyrate (NaB) and trans-resveratrol (RV). IL-6 was included to mimic the inflammatory tumor microenvironment. The anticancer properties of both NaB and RV, individually or in combination, were tested in wound healing scratch assay, 3D-spheroid forming assay, and immunofluorescence double-staining of epithelial to mesenchymal transition markers and β-catenin. Wound healing assays showed that NaB and RV combination achieved significant inhibition compared to individual inhibitory effects. NaB and RV reduced HCT116 cell migration and proliferation by promoting β-catenin retention in the cytoplasm bound to E-cadherin. In 3D spheroid assays, combination of NaB and RV resulted in higher reduction of spheroid growth than the reducing effects of NaB and RV alone. Immunofluorescence revealed increased cytoplasmic retention of β-catenin bound to E-cadherin and decreased nuclear β-catenin activity, confirmed by downregulation of SNAIL and Cyclin D1. These findings demonstrate a synergistic effect of NaB and RV in inhibiting CRC cell proliferation and migration, supporting their potential as adjuvant dietary interventions in CRC management. These preliminary *in vitro* data consistently demonstrate the synergism between butyrate and resveratrol in inhibiting CRC cell proliferation and migration, supporting the view that postbiotics and nutraceuticals have great potential as adjuvant and complementary therapeutic agents in CRC treatment.

Keywords: colorectal cancer; microbiota; postbiotics; nutraceuticals; dietary supplements.

1. Introduction

According to the GLOBOCAN 2018 statistics, colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of cancer-related deaths worldwide, despite improvements in early detection and advancements in therapeutic treatments [1]. Various widespread factors play a significant role in the development of CRC, including physical inactivity, smoking, western diet, alcohol consumption, and obesity [2]. CRC is a heterogeneous disease at genetic and epigenetic levels, driven by mutations that occur in the epithelial cells of the large intestine [3,4]. Sporadic CRC, which accounts for up to 80-85% of the cases, originates in the large intestine from polyps and adenomas that eventually evolve into adenocarcinoma following the accumulation of mutations and epimutations in oncogenes and tumor suppressor genes over a period of approximately 10 to 15 years [5]. Typically, early Adenomatous polyposis coli (*APC)* mutation occurs in colorectal adenomas, which progress to carcinoma by generating truncated gene products, which activate Wnt signalling and deregulate multiple cellular processes [6]. *APC* mutations disrupt the formation of the degradation complex of β-Catenin, a transcription factor of the Wnt pathway that transcribes genes involved in cell proliferation, epithelial-to-mesenchymal transition (EMT), and matrix remodeling enzymes, thus contributing to cancer cell proliferation, invasion, and metastasis [7,8]. Point mutations in the β-catenin gene can prevent ubiquitination and proteasomal degradation of the protein [9]. Diet is the most important modifiable factor risk for CRC, primarily due to its impact on intestinal inflammation and microbiome composition, which greatly influence epithelial intestinal homeostasis and integrity [10-13].

The gut microbiota plays a pivotal role in human health, particularly through its ability to ferment dietary fibers into short-chain fatty acids (SCFAs), such as butyrate, propionate, and acetate. Butyrate is a key postbiotic produced in the colon through the anaerobic fermentation of indigestible polysaccharides (prebiotics) such as dietary fibers and resistant starch. Among butyrate-producing microorganisms, *Faecalibacterium prausnitzii* is considered one of the

most significant [14]. Postbiotics like butyrate have been shown to contrast colorectal cancer cells through stimulation of the immune response, by promoting a tumor suppressive microenvironment, by inhibiting cancer cell proliferation and migration, by dampening inflammation and oxidative stress, and by inducing apoptosis of cancer cells and autophagy [13,15-18]. Interestingly, butyrate can induce autophagy as an alternative pathway for degradation of β-Catenin in CRC cells bearing mutations in *APC* or *CTNNB1* genes [19]. Several dietary compounds, including soy isoflavones, curcumin, green tea polyphenols, resveratrol, 3,3′-diindolylmethane, and lycopene, have demonstrated the potential to prevent, slow down, or even reverse colorectal carcinogenesis [20,21].

Resveratrol (3,4',5-trihydroxy-trans-stilbene; RV) is a non-flavonoid polyphenol and phytoalexin naturally found in peanuts, grapes, pines, and berries, where it serves as a defense mechanism against pathogen infections [22]. Trans-RV has been reported to exhibit anti-tumor activities in colorectal carcinogenesis [23-26]. Further, RV has been shown to suppress TGF-β1-induced EMT by increasing E-cadherin expression while decreasing the expression of vimentin, fibronectin, and EMT-inducing transcription factors Slug and Snail [27]. Resveratrol alleviated H_2O_2-induced epithelial barrier disruption in the Caco-2 cell model of oxidative stress by inhibiting occludin tyrosine phosphorylation. Additionally, it reduced IL-6-induced hyperpermeability in the inflamed cell model by downregulating claudin-2 expression [28]. Here we investigate whether and through which mechanisms butyrate and resveratrol elicit a synergistic effect in inhibiting CRC cell proliferation and migration in the presence of the pro-inflammatory cytokine IL-6, a context mimicking the inflammatory tumor microenvironment of CRC.

2. Materials and methods

2.1 Cell culture

Human colorectal cancer HCT116 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM, cod. D5671; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heated-inactivated fetal bovine serum (FBS, cod. ECS0180L; Euroclone, Milan, Italy), 1% Glutamine (cod. G7513; Sigma Aldrich) and 1% Penicillin/Streptomycin (cod. P0781; Sigma-Aldrich). Cells were grown in standard conditions (37°C, 95 v/v % air: 5 v/v % CO_2).

2.2 Reagents

Interleukin-6 (IL-6, cod. 11340066; Immunotools, Friesoythe, Germany) was dissolved in sterile water and used at the final concentration of 50 ng/ml. Sodium butyrate (NaB, cod. B5887; Sigma-Aldrich) was dissolved in sterile water and used at the final concentration of 0.1 and 2 mM. Resveratrol (RV, cod. R5010; Sigma-Aldrich) was dissolved in DMSO and used at a final concentration of 10, 20, and 100 μM.

2.3 Antibodies

The following primary antibodies were used for immunofluorescence: rabbit anti-N-cadherin (1:50, cod. 4061S; Cell Signaling Technologies, Danvers, MA, USA); mouse anti-E-cadherin (1:50, cod. 610404; BD Biosciences, Franklin Lakes, NJ, USA), rabbit anti-β-catenin (1:500, cod. PA5-77934; Invitrogen, Paisley, UK).

2.4 Immunofluorescence

Cells were seeded onto sterile coverslips at a density of 25,000 cells/cm^2, let to adhere, and treated as reported. At the end of the experiment, the coverslips were fixed within ice-cold methanol, permeabilized with 0.2% Triton-PBS, and incubated overnight at 4°C with specific primary antibodies dissolved in 0.1% Triton-PBS + 10% FBS. The following day, coverslips were incubated for 1 hour at room temperature with secondary antibodies (diluted in 0.1% Triton-PBS + 10% FBS), followed by AlexaFluor488-conjugated goat-anti rabbit IgG (1:000, cod. A32731; Invitrogen) or AlexaFluor555-conjugated goat-anti mouse IgG (1:1000, cod. A32727; Invitrogen), as appropriate. Nuclei were stained with UV fluorescent dye DAPI (40,6-diamidino-2-phenylindole). Coverslips were mounted onto glasses using SlowFade reagent (cod. S36936; Life Technologies, Paisley, UK) and imaged with a fluorescence microscope (Leica DMI6000, Leica Microsystems, Wetzlar, Germany).

2.5 Wound healing migration assay

Cells were seeded in 35mm Petri dishes at a density of 40,000 cells/cm^2, cultured until confluence, and then treated as indicated. The cell monolayer was scratched with a sterile pipette yellow tip to produce a straight line, and the debris was washed out with PBS. Medium and treatments were renovated every 24 hours. The open gap was photographed with a camera at phase contrast microscope (magnification 5×, Zeiss AXIOVERT 40CFL, Zeiss, Oberkochen, Germany) at the indicated times. The rate of healing was estimated by ImageJ software, calculating the area free of cells. Data are calculated for three different fields per each condition.

2.6 3D-spheroid forming assay

Cells were cultured in 35mm Petri dishes coated with poly(2-hydroxyethylmethacrylate) (polyHEMA; cod. P3932; Sigma Aldrich) to prevent cell adhesion. The polyHEMA stock solution was diluted in 95% ethanol to obtain a working solution of 120 mg/ml. Petri dishes were coated with this solution (working concentration: 5mg/ml), left under a biological hood to completely dry, and then stored at room temperature until use. Cells were seeded at a density of 500,000 cells/Petri and treated according to the experimental conditions. The growth of spheroids was monitored by taking pictures with a phase-contrast microscope at each time point.

The quantification of the dimensions of the spheroids was analyzed with ImageJ software.

2.7 RNA Isolation and Quantitative PCR

HCT116 cells were plated in 60mm Petri dishes at a density of 50,000 cells/cm^2 and treated as indicated. The total RNA was extracted from the cells using TRIzol reagent (cod. T9424, Sigma-Aldrich). The mRNA was then reverse-transcribed into complementary DNA (cDNA) using the Revert Aid First Strand cDNA Synthesis Kit (cod. K1622, Thermo-Scientific, Waltham, MA, USA). The cDNA was then amplified by PCR in the presence of recombinant Taq DNA polymerase (cod. 10342-020, Invitrogen). The PCR products were then analyzed by agarose gel electrophoresis. The sequences of the primers used were as follows: Cyclin D1 (forward 5'-CACACACACACACACAAACC-3' and reverse 5'-CCTCCCTTCAACACTTCCTAAA-3'); SNAIL (forward 5'-CCTTCGTCCTTCTCCTCTACTT-3' and reverse 5'-GGCACTGGTACTTCTTGAGATC-3'); β-actin (forward 5'-GATCAAGATCATTGCTCCTCCTGAGCGCA-3' and reverse 5'-GTCTCAAGTCAGTGTACAGGTAAGCCCT-3').

2.8 IncuCyte® Scratch Wound Assay

HCT116 cells (20,000 cells/well) were seeded into an IncuCyte® Imagelock 96-well plate (cod. BA-04855; Sartorius, Göttingen, Germany) and incubated in a standard incubator for 24 hours. The following day, the IncuCyte® 96-well wound maker was used to create wounds in all wells. After scratching, the media was aspirated from each well, and the wells were washed with culture media to remove debris and prevent detached cells. After washing, the media and treatments were added to each well as indicated. The plate was then incubated in the IncuCyte® live-cell analysis system. Imaging was scheduled every 24 hours for six days using phase contrast at 10× magnification.

2.9 IncuCyte® Spheroid Formation Assay

HCT116 cells (2,500 cells/well) were seeded in a 96-well ultra-low attachment plate. The plate was centrifuged at 1,000 rpm for 10 minutes at room temperature to collect the cells at the bottom, then placed in the IncuCyte® live-cell analysis system. After three days, the spheroids reached the desired size, and treatment was initiated according to the experimental conditions. Imaging was scheduled for every 24 hours over six days using phase contrast at 10× magnification. Study design for evaluating the effects of sodium butyrate and resveratrol on IL-6-induced migration and proliferation of HCT116 colorectal cancer cells is given as figure 1.

2.10 Statistics

Statistical analysis was performed using GraphPad Prism 5.0 software. ANOVA and t-tests were used, depending on the experiment conducted. Significance was considered as follows: * $p < 0.05$. Data are reported as mean of triplicates ± SD.

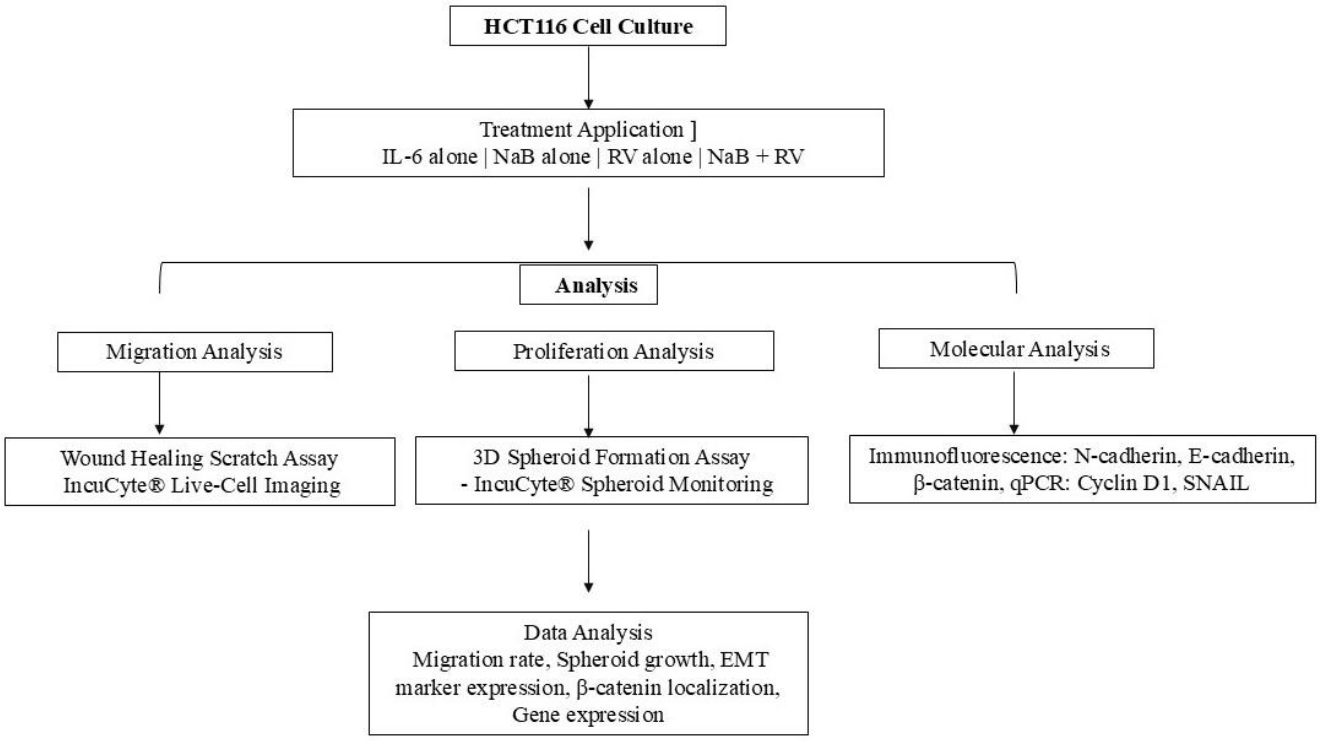


Figure 1. Schematic representation of the study design.

3. Results

3.1 Butyrate and Resveratrol Counteract IL-6-Induced Colorectal Cancer Cell Migration by Downregulating EMT Markers

To evaluate the role of sodium butyrate (NaB) and resveratrol (RV) on HCT116 cell migration, we performed a wound healing scratch assay and monitored the closure rate over 72 hours. The wound healing assay shows that IL-6 enhanced cell migration, leading to approximately 90% wound closure within 72 h, whereas either NaB or RV markedly reduced HCT116 migration, also in the presence of IL-6 (Figure 2A). Since epithelial-to-mesenchymal transition (EMT) endows cells with migratory and invasive properties, we investigated whether NaB and RV contrast migration by modulating EMT marker expression in the cells at the migration front. Immunofluorescence staining shows that NaB and RV strongly reduced N-cadherin, a metastatic marker, and increased E-cadherin, an epithelial marker crucial for the maintenance of the epithelial phenotype (Figure 2B).

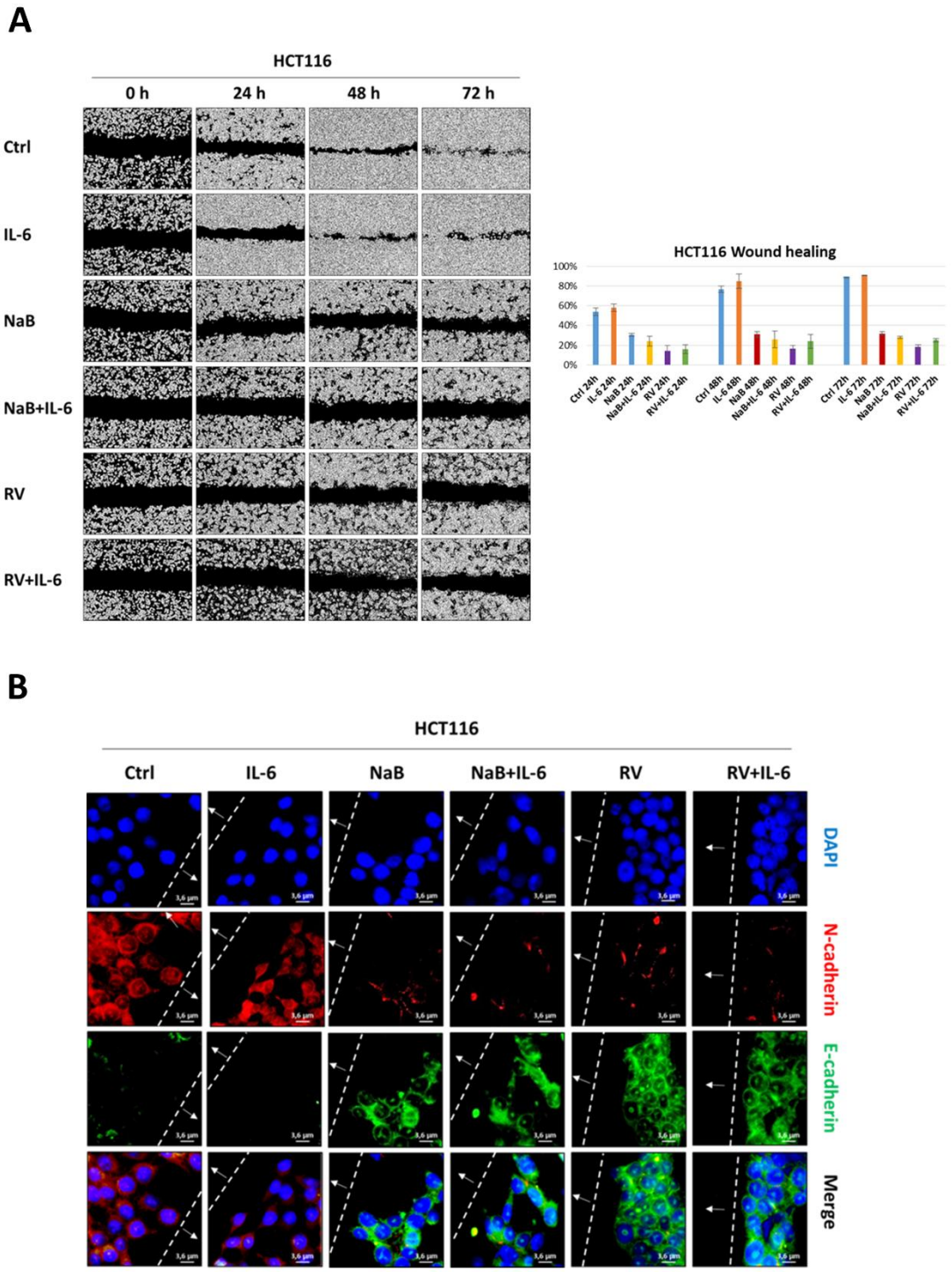


Figure 2. NaB and RV inhibit IL-6-induced cell migration. (A) HCT116 cells were plated, scratched, and treated with 2 mM NaB and 100 µM RV alone or in combination with 50 ng/ml IL-6. The treatment was renewed every 24 hours. The

images were acquired with a phase-contrast microscope at 0, 24, 48, and 72 hours. The rate of wound closure for each time is represented by the chart and is calculated on triplicates with ImageJ software. Data are expressed as average +/- S.D. (B) HCT116 cells were plated on coverslips, scratched, and treated as previously described. The treatment was renewed every 24 hours. Following 48 hours of incubation, coverslips were fixed and stained for double-staining N-cadherin (red) and E-cadherin (green). Images were acquired with the fluorescent microscope, focusing on the cells near the migration front. Representative images of experiments in duplicate are shown.

3.2 Sodium Butyrate and Resveratrol Strongly Reduce the Growth of Colorectal Cancer 3D Spheroids

To investigate the effects of NaB and RV on cancer cell proliferation, we set a 3D spheroid model that better mimics the *in vivo* tumor growth. We monitored the ability of HCT116 cells to form spheroids for up to 5 days. We found that IL-6 increased 3D spheroid dimensions, whereas NaB and RV markedly reduced them, even in the presence of IL-6. The analysis confirms the reduction in spheroid diameter in NaB- and RV-treated conditions compared to the IL-6-treated cultures (Figure 3). This suggests that NaB and RV impair colorectal cancer cell proliferation and limit spheroids' growth, counteracting the pro-tumorigenic effects of IL-6.

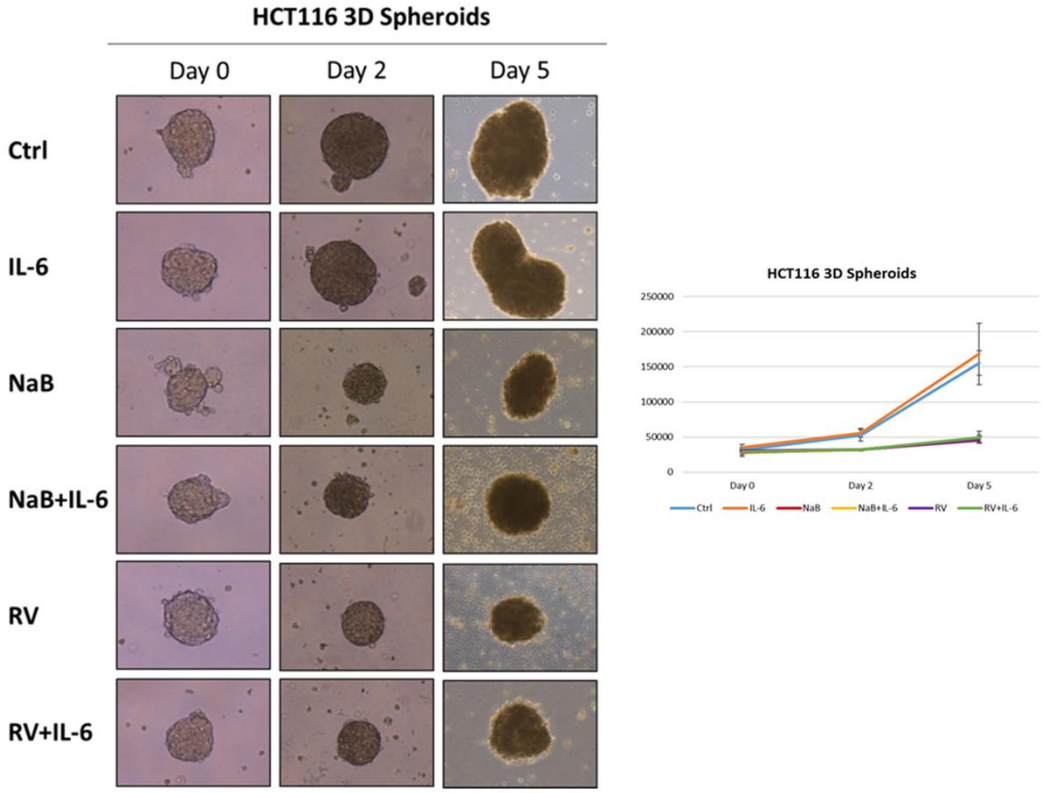


Figure 3. NaB and RV inhibit the IL-6-promoted growth of 3D colorectal spheroids. Cells were plated on polyHEMA-coated Petri dishes and treated with 2 mM NaB and 100 µM RV alone or in combination with 50 ng/ml IL-6. Representative images were acquired with a phase-contrast microscope on day 0, 2 and 5. Relative quantification of spheroid diameter calculated on ten spheroids randomly chosen is reported. The experiments were replicated two times with similar results. Data are expressed as average +/- S.D.

3.3 Butyrate and Resveratrol Reduce the Nuclear Translocation of β-catenin Promoting its Interaction with E-cadherin

The upregulation of the Wnt/β-catenin pathway plays a critical role in CRC progression. In physiological conditions, when its transcriptional activity is repressed, β-catenin is either sequestered at the plasma membrane through its interaction with E-cadherin or targeted for degradation via the APC complex. However, in cancer, β-catenin constitutively translocates to the nucleus, where it acts as a transcriptional activator, promoting the expression of genes involved in migration and proliferation [29].

Given our previous findings that NaB and RV reduce migration and proliferation, decrease N-cadherin, and increase E-cadherin levels, we investigated whether these effects are mediated by inhibition of the Wnt/β-catenin signaling pathway. To assess this, we performed immunofluorescence staining to analyze β-catenin localization. We observed that IL-6 promotes β-catenin perinuclear position, suggesting its transcriptional activity. On the contrary, NaB and RV promote β-catenin cytoplasmic co-localization with E-cadherin (yellow signal), even in the presence of IL-6 (Figure 4A).

To further confirm the transcriptional activity of β-catenin in the different conditions, we analyzed the mRNA expression levels of SNAIL, a key regulator of migration, and Cyclin D1, a proliferation-associated gene. Since nuclear β-catenin acts as a transcriptional activator, its presence in the nucleus should correlate with increased expression of these target genes. Our results show that IL-6 treatment upregulates both SNAIL and Cyclin D1, supporting the observation that β-catenin is transcriptionally active. In contrast, NaB and RV treatment downregulate these transcripts, even in the presence of IL-6, indicating that β-catenin nuclear translocation is impaired and its transcriptional activity is reduced (Figure 4B,C).

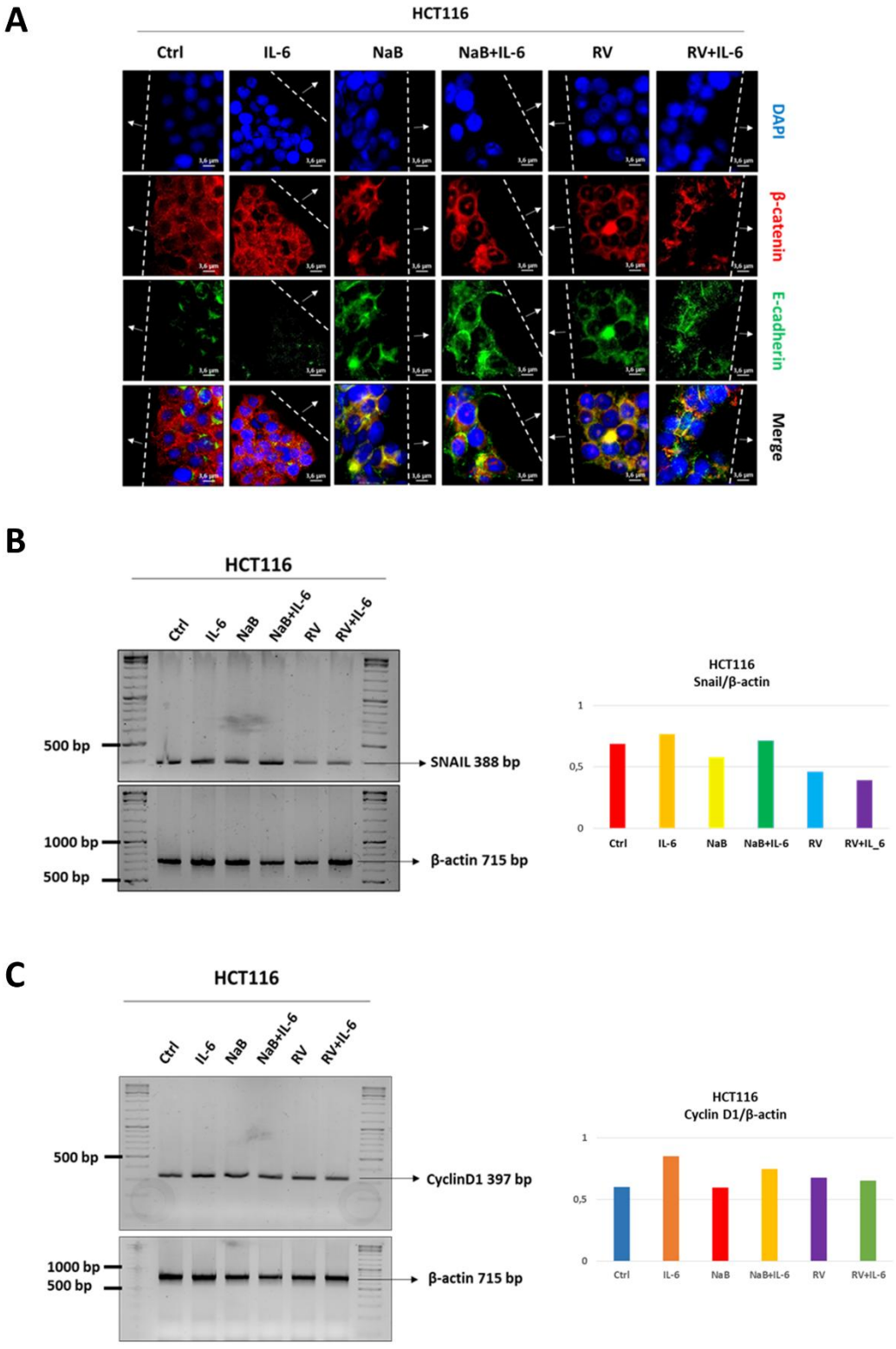
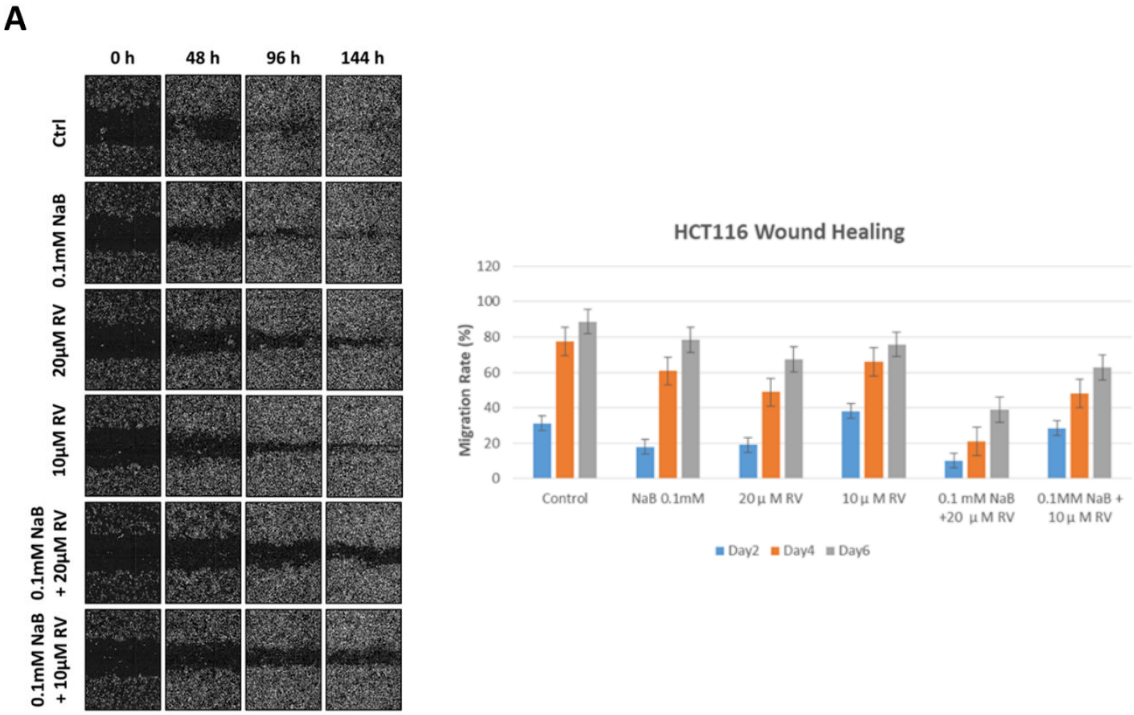


Figure 4. NaB and RV promote β-catenin and E-cadherin interaction, inhibiting IL-6-induced β-catenin transcriptional activity
(A) HCT116 cells were plated on coverslips, scratched, and treated with 2 mM NaB and 100 µM RV alone or in combination with 50 ng/ml IL-6. The treatment was renewed every 24 hours. Following 48 hours of incubation, coverslips were fixed and stained for double-staining β-catenin (red) and E-cadherin (green). Images were acquired with the fluorescent microscope, focusing on the cells near the migration front. Representative images of two replicates are shown. (B,C) HCT116 cells were cultured in 60 mm Petri dishes and treated as described above for 48 hours. The quantitative PCR was performed to monitor the mRNA expression of β-catenin targets SNAIL (B) and Cyclin D1 (C). The graph reports the quantification of the bands detected in agarose gel electrophoresis in one pilot experiment.

3.4 Butyrate and Resveratrol Synergize in Reducing the Migration and Proliferation of Colorectal Cancer Cells

NaB and RV have shown promising effects in inhibiting cancer cell migration and growth, but the combination of lower doses may lead to enhanced therapeutic outcomes. By targeting different aspects of tumor biology, we hypothesized that NaB and RV could synergize and elicit a more potent response than when administered separately. We tested this hypothesis by wound-healing assay and 3D growth assay. The data confirms that NaB and RV can inhibit the motility (Figure 5A) and spheroid growth (Figure 5B) of CRC cells when combined at very low concentrations. In detail, the best combinatory treatment appears to be 0.1mM NaB and 20µM RV, which effectively reduces the rate of healing and stops the growth of 3D spheroids.



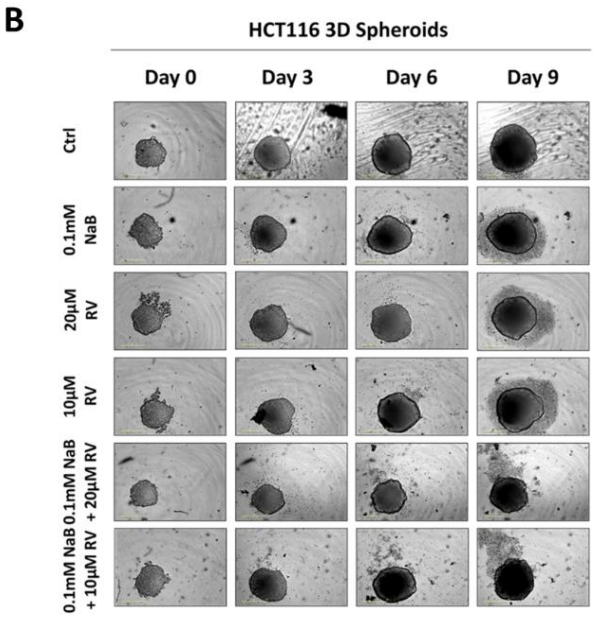


Figure 5. Synergistic effect of NaB and RV in reducing migration and proliferation of CRC cells. (A) HCT116 cells were seeded into an IncuCyte® Imagelock 96-well plate. After scratching, the cells were treated as reported in the figure panel. Imaging was scheduled every 24 hours for 6 days using phase contrast at 10× magnification. The rate of wound closure for each time is represented by the chart and is calculated on triplicates with ImageJ software. Data are expressed as average +/- S.D. (B) HCT116 cells were seeded in a 96-well ultra-low attachment plate. After 3 days, the spheroids reached the desired size, and treatment was initiated according to the experimental conditions. Imaging was scheduled for every 24 hours over 6 days using phase contrast at 10× magnification. Images are representative of the experiment performed one time in duplicate with similar results.

4. Discussion and conclusion

Colorectal cancer (CRC) is a complex and heterogeneous disease, with multiple genetic and epigenetic alterations driving its progression. Individuals with predisposing mutations, such as those in the *APC* and *CTNNB1* gene, often accumulate somatic mutations over time, leading to the transformation of normal epithelial cells into malignant cells [30]. Chemotherapy remains a cornerstone in CRC management, particularly in metastatic disease, with regimens such as FOLFOX (oxaliplatin, fluorouracil, leucovorin) and FOLFIRI (irinotecan, fluorouracil, leucovorin) commonly used. Additionally, targeted therapies with monoclonal antibodies that focus on specific signaling pathways and with immune checkpoint inhibitors are becoming increasingly integral to treatment. Still, as these treatments are not completely successful, there is a need to implement the prevention and to complement these therapies with (neo)adjuvant dietary supplements. In recent years, epigenetic therapy has emerged as a promising approach in nutraceutical development, particularly for cancer prevention. Advances nutrigenomics, nutrigenetics, and nutraceutical research have enabled the identification of superfoods capable of modulating gene expression in a beneficial way [31]. The aberrant activation of Wnt/β-catenin signaling pathway is a well-established hallmark of CRC, and factors involved in its regulation remain attractive for therapeutic interventions [32].

EMT is characterized by a “cadherin switch” in which epithelial E-cadherin is replaced by mesenchymal N-cadherin, weakening cell-cell adhesion and facilitating migration and invasion [33]. The Wnt/β-catenin signaling contributes to EMT. Wnt binding to its receptor stabilizes β-catenin, allowing its nuclear translocation and activation of target genes involved in cell migration and invasion such as *SNAIL* and *TWIST1* [34]. The resulting loss of β-catenin interaction with E-cadherin can lead to β-catenin nuclear translocation [35, 36]. The findings presented in this study demonstrate the potential of NaB and RV as adjuvant agents for counteracting CRC progression. Our results provide evidence for their ability to inhibit key processes involved in tumorigenesis, including cell migration, proliferation, and EMT, alongside the modulation of the Wnt/β-catenin signaling pathway. Furthermore, the synergistic effects of NaB and RV at lower concentrations suggest a promising strategy for enhancing therapeutic efficacy while minimizing potential adverse effects.

The wound healing assay provided insight into the migratory behavior of HCT116 cells. The inflammatory cytokine IL-6 significantly enhanced CRC migration. However, both NaB and RV were able to markedly counteract the wound closure even in the presence of IL-6. This anti-migratory effect was further supported by the modulation of EMT markers. Immunofluorescence staining showed that NaB and RV treatment (alone) led to increased E-cadherin expression and decreased N-cadherin levels, indicating a reversal of the EMT phenotype promoted by IL-6. NaB and RV also exerted inhibitory effects on CRC growth of 3D model spheroids, even in the presence of the inflammatory cytokine, which mimics a permissive tumor microenvironment.

The role of the Wnt/β-catenin pathway in CRC progression is pivotal, with its dysregulation promoting the nuclear translocation of β-catenin and subsequent transcriptional activation of genes involved in migration and proliferation. In our study, immunofluorescence analyses revealed that NaB and RV treatment promoted the cytoplasmic co-localization of β-catenin with E-cadherin, suggesting that both compounds inhibit β-catenin nuclear translocation. This was supported by the downregulation of β-catenin target genes *SNAIL* and *Cyclin D1*, which promote migration and proliferation, respectively. These findings confirm that NaB and RV exert their anti-tumor effects by modulating the Wnt/β-catenin pathway.

Notably, here we also demonstrated the synergistic effect of NaB and RV. The compounds, used at the minimal doses, combined ability to counteract CRC cell migration and proliferation. This combination approach is particularly promising as it may allow for the use of lower concentrations of NaB and RV, thereby reducing the risk of adverse side effects. This synergism further highlights the relevance of dietary bioactive compounds in modulating oncogenic pathways and attenuating CRC progression. This is particularly relevant for CRC, where resistance to conventional therapies and the risk of metastasis remain significant challenges. The therapeutic relevance of these bioactive compounds in CRC is particularly noteworthy, as they

may offer a novel, multifaceted approach to fighting this aggressive and heterogeneous disease. We acknowledge that this study is still preliminary in various aspects and the findings here reported should rather be considered as "proof-of-concept". There are a few limitations that need to be addressed in future studies, the most important being the fact that only one cell line has been used and to generalize the conclusion it is mandatory to extend the same analysis in other cell lines with different genetic backgrounds. In separate studies, we have tested the anticancer effects of postbiotics and of resveratrol individually in several colorectal cancer cell lines including HCT116, HT29, SW620 and DLD-1 (16,18,19,24,25). Here, the HCT116 colorectal cancer cell line has been selected because, relevantly for the present study, these cells carry the mutation in the β-catenin gene (*CTNNB1*), offering a valuable model for studying β-catenin gain-of-function effects on CRC progression [37]. Regarding the proposed mechanism, we could add a nuclear/cytoplasmic protein fractionation assay to confirm the nuclear-cytoplasmic shuttling of β-catenin. We acknowledge the importance of including additional cells lines in future studies, and particularly we will extend such analysis to SW620 cells that bear a mutation in the *APC* gene. In the future, the *in vitro* model should be implemented with heterogenic spheroids that include also stromal cells (namely, CAFs and TAMs), possibly using microfluidic systems to resemble the dynamic circulation of cells (thus mimicking the metastatic process) and soluble factors (such as cytokines, postbiotics, nutraceuticals). Although preliminary, the present data hold great translational potential where the synergy of diet-driven manipulation of the microbiota and nutraceutical supplementation could modulate key oncogenic signaling pathways and autophagy, and sensitize colorectal tumors to conventional therapies, thereby contributing to a more integrative and personalized approach to colorectal cancer prevention and treatment. Thus, despite the intrinsic limitations of our study, considering the total absence of unwanted side-effects and the optimal beneficial/risk ratio, we strongly advise making use of appropriate probiotics, postbiotics, and phytochemicals to implement the management of colorectal cancer for both preventive and curative purposes.

This preliminary study demonstrates that NaB and RV individually and in combination can reduce the IL-6 induced migration and proliferation of HCt116 colorectal cancer cells by modulating EMT markers and β-catenin signaling. The results suggest potential synergistic effects at low concentrations. However, these findings as based on invitro experiments and further studies with in vivo models are required to confirm these effects and to understand the underlying mechanisms.

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