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RESEARCH

Berberine enhances the therapeutic effect of 5-fluorouracil in Caco-2 colorectal adenocarcinoma cells by alleviating inflammation and inducing apoptosis

Berberin inflamasyon baskılanması ve apoptoz indüksiyonu ile Caco-2 kolorektal adenokarsinom hücrelerinde 5-florourasil'in terapötik etkisini arttırır

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Abstract

Purpose: This study aims to investigate whether berberine (BBR) and 5-fluorouracil (5FU), which forms the backbone of chemotherapy, have a synergistic effect on colorectal adenocarcinoma cells (Caco-2) and what mechanisms might be behind this.

Materials and Methods: Cell viability was determined by MTT and the combination index (CI) by *Chou-Talalay* method. Apoptosis and inflammation-related proteins (Bcl-2, p53, IL-6, TNF-α, MMP-9) were measured by ELISA.

Results: The IC_{50} values of BBR and 5FU were found to be 280 μM and 20 mM for 24 h. The combination treatment showed synergistic cytotoxicity, both of which were more pronounced at IC_{50} (CI=0.143). 5FU+BBR showed a synergistic apoptotic effect by decreasing Bcl-2 and increasing p53 (0.712-fold decrease in Bcl-2 and 2.650-fold increase in p53 compared to 5FU). The increase in IL-6 and TNF-α by 5FU was significantly suppressed by 5FU+BBR (0.733-fold and 0.485-fold). Although there was no significant difference in MMP-9 in 5FU compared to control, 5FU+BBR significantly decreased MMP-9 (0.601-fold).

Conclusion: The results show the enormous potential of BBR in combination with 5FU for the chemotherapy of colorectal carcinoma through apoptosis induction, inflammation inhibition and metastasis inhibition.

Keywords:. berberine (BBR), 5-fluorouracil (5FU), synergism, Caco-2, apoptosis, MMP-9

Öz

Amaç: Bu çalışma, berberin (BBR) ile kemoterapinin omurgasını oluşturan 5-fluorourasil (5FU)'in Caco-2 kolorektal adenokarsinoma hücreleri üzerinde sinerjik etki gösterip göstermediğini ve olası mekanizmalarını araştırmayı amaçlamaktadır.

Gereç ve Yöntem: Hücre canlılığı MTT yöntemiyle ve kombinasyon indeksi (CI) *Chou-Talalay* yöntemiyle belirlendi. Apoptoz ve inflamasyonla ilişkili proteinlerin (Bcl-2, p53, IL-6, TNF-α, MMP-9) konsantrasyonları ELISA ile ölçüldü.

Bulgular: BBR ve 5FU'nun IC_{50} değerleri 24 saat için 280 μM ve 20 mM olarak bulundu. Kombinasyon tedavi, her ikisinin de IC_{50} konsantrasyonlarında daha belirgin olmak üzere, sinerjik sitotoksik etki gösterdi (CI= 0.143). 5FU+BBR, Bcl-2'yi azaltarak ve p53'ü artırarak sinerjistik apoptotik etki gösterdi (5FU ile karşılaştırıldığında, Bcl-2 için 0.712 kat azalış ve p53 için 2.650 kat artış). Ayrıca, 5FU ile artan IL-6 ve TNF-α düzeyleri, 5FU+BBR tedavisi ile belirgin şekilde baskılandı (0.733 kat ve 0.485 kat). Ek olarak, 5FU ile tedavi edilen hücrelerde kontrol grubuna göre MMP-9 düzeylerinde anlamlı bir fark yokken, kombine tedavi MMP-9 düzeylerini belirgin şekilde (0.601 kat) azalttı.

Sonuç: Bulgular, BBR'nin 5FU ile birlikte kolorektal kanseri kemoterapisi için apoptoz indüksiyonu, inflamasyon baskılaması ve metastaz inhibisyonuyla muazzam potansiyelini ortaya koymaktadır.

Anahtar kelimeler: Berberin (BBR), 5-fluorourasil (5FU), sinerjizm, Caco-2, apoptosis, MMP-9

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INTRODUCTION

Colorectal cancer is the third most common type of cancer worldwide and the second most common cause of cancer-related death[1]. The subtypes have different prognoses and therapeutic responses due to different hereditary and biological characteristics[2]. Despite enormous progress in the diagnosis and treatment of colorectal cancer, the prognosis remains poor. Current treatment includes surgery, radiotherapy, and chemotherapy[3,4]. Chemotherapy is an essential and indispensable option for unresectable colorectal tumors. Common cytotoxic chemotherapeutic agents include 5-fluorouracil (5FU), irinotecan, oxaliplatin, and raltitrexed, as well as vascular endothelial growth factor inhibitors (such as bevacizumab, regorafenib) and epidermal growth factor receptor inhibitors (such as cetuximab, panitumumab)[5]. Despite the superior anticancer efficacy of 5FU, which is the backbone of colorectal cancer chemotherapy, its rapid metabolism, short half-life, low bioavailability, and high non-selective cytotoxicity limit its treatment[5,6]. On the other hand, the disruption of the mucosal barrier by chemotherapeutic agents due to the inflammatory response causes gastrointestinal toxicity, which is a dose-limiting toxicity. This deterioration creates a negative basis for the next treatment steps[7]. There is an urgent need to develop safe and highly effective innovative strategies to overcome these drawbacks, increase treatment efficacy and reduce the economic burden[8].

Traditional Chinese medicine is widely accepted in China as a complementary and alternative therapy for many cancer patients, including colorectal cancer[9]. Berberine (BBR), a typical derivative of Chinese herbal medicine and also known as Coptis rhizome, stands out as an effective, safe, cost-effective and promising drug candidate for the treatment of colorectal cancer[10-15].

BBR has been reported to suppress the development and progression of colorectal cancer by modulating various signaling pathways[10,16]. These pathways include gene expression (microRNAs, long non-coding RNAs, mRNAs)[17-19], growth factors (EGFR)[20], cell cycling[21], and various signaling pathways (AMPK/JAK2/signal transducer and transcription 3 activator STAT3, cyclooxygenase-2/progtaglandin E2 signaling pathways, Wnt/β-catenin)[11,22]. Moreover, the regulation of intestinal flora and mucosal barrier function by BBR shows its growing potential for targeted cancer therapy[23,24]. Furthermore, BBR has been shown to potentiate its anticancer effects by sensitizing various cancer cells, including colorectal cancer, to radiotherapy and chemotherapeutic agents[25,26]. On the other hand, BBR has also been reported to significantly reduce the anticancer effects of chemotherapeutic agents, especially at low doses, including 5FU, camptothecin, and paclitaxel[27]. While the efficacy of BBR in combination with chemotherapy is still uncertain, there is a growing number of studies suggesting that it may have a synergistic effect. The possible mechanism underlying this synergistic effect is not yet fully understood, but ROS-mediated regulation of apoptosis and inflammatory response may serve as an attractive therapeutic target[14,28-32]. Our study will provide a mechanistic perspective to the literature on the status of the potential synergistic effect of 5FU and BBR. In this study, we investigated whether 5FU and BBR synergistically enhance the cytotoxic effect on Caco-2 cells, as well as possible modulations of apoptosis and inflammatory response underlying this potential synergistic effect.

MATERIALS AND METHODS

Chemicals

5FU (250 mg/ml, for intravenous infusion) was obtained from Kocak Pharma (Tekirdag, Turkey). BBR (cat. no. 10006427) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Dulbecco's Modified Eagle Medium (DMEM), dimethyl sulfoxide (DMSO), Dulbecco's phosphate-buffered saline (PBS), glutamine, penicillin-streptomycin solution, thiazolyl blue tetrazolium bromide (MTT), and trypan blue were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Heat-inactivated fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco (Thermo Fisher Scientific Inc, Waltham, MA, USA).

Cell culture

The Caco-2 cell line (American Type Culture Collection, Rockville, MD, USA, No.: HTB-37) was obtained from the Medical Biochemistry Research Laboratory of the Faculty of Medicine, Afyonkarahisar Health Sciences University. All experimental applications were performed in this laboratory by researchers specializing in biochemistry, pharmacology and toxicology. Since we worked with commercially available cell lines, no

ethics committee approval was required for this study. Caco-2 cells were maintained in DMEM at 37°C and 5% CO_2. All media were supplemented with 10% FBS and 1% penicillin/streptomycin solution. After 80-90% attachment, cells were passaged with 0.25% trypsin-EDTA solution. Subcultures were established in the same culture medium. Caco-2 cells (10000 cells/well) were seeded in 96-well plates and allowed to adhere overnight.

Drug treatment

The cytotoxicity of 5FU and BBR was evaluated after 24 hours of treatment in alone and combination studies. First, the half-maximal inhibitory concentration (IC_{50}) of both agents alone was determined in Caco-2 cells. 5FU alone was tested at concentrations of 6.25, 12.5, 25, 50, 100, and 200 mM. BBR alone was tested at concentrations of 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µM. In addition, cytotoxicity studies were performed for the combination of 5- FU (with IC_{50} and $IC_{50}/2$) and BBR (IC_{50}x2, IC_{50} and $IC_{50}/2$). The concentrations with the strongest synergistic cytotoxic effect were selected for the mechanistic experimental groups. The control cells were treated with 0.5% DMSO.

Cell viability assay (MTT)

The effects of monotherapy and combined therapy with 5FU and BBR on the viability of Caco-2 cells were investigated using the MTT assay[33]. This method was also used in our previous combination study[28] and the same methodology was used in the current study. All experiments were performed at 3 different time points. GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA) was used to determine the IC_{50} values of 5FU and BBR from the MTT data. Concentration-response curves were plotted against drug concentrations on a logarithmic scale by nonlinear regression analysis of cell viability fractions normalized to control cell viability.

Determination of synergy

CompuSyn (ComboSyn, Inc., New York, NY, USA) was used to measure the synergism between 5FU and BBR. The combination index (CI), which is a quantitative representation of the pharmacologic interactions of the combinations at a non-constant dose rate, was calculated. To evaluate the synergism between this reference model, introduced as the *Chou-Talalay*[34] method and the drug combinations, a graph is constructed in which the CI is plotted on the y-axis as a function of the efficiency (Fa) on the x-axis. According to this method, CI < 1 means synergism, CI=1 means additive interaction, and CI > 1 means antagonism.

Enzyme-linked immunosorbent assay (ELISA) for the detection of apoptotic and inflammatory proteins

Analysis of the levels of Bcl-2, p53, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and matrix metalloproteinase (MMP)-9 in the supernatants was performed using commercially available ELISA kits (Bioassay Technology Laboratory, Birmingham, UK). Optical density was measured using a microplate reader. The protein concentrations (pg/mg) of the samples were calculated using graphs obtained from standard concentrations.

Statistical analysis

Numerical data are expressed as mean ± standard deviation (n=3). The software program SPSS 20.0 (SPSS Inc., IBM, Chicago, IL, USA) was used for all statistical analyzes. The mean values of the data (MTT cytotoxicity analyzes and Bcl-2, p53, IL-6, TNF-α, MMP-9 analyzes) were compared using one-way analysis of variance (ANOVA). Post-hoc analysis of differences between groups was performed using Tukey's HSD test. P-values below 0.05 were considered statistically significant.

RESULTS

To investigate the antiproliferative effect of BBR, Caco-2 cells were treated with BBR at a wide range of concentrations (15.62-1000 µM) over a 24-hour period. Cell viability was assessed after treatment using the MTT assay, which is commonly used in pharmacological studies and provides important information on mitochondrial metabolism and cellular protein content. MTT results showed a significant decrease in the viability of BBR-treated cells at concentrations above 250 µM, and the IC_{50} value was reported to be 281.4 µM (Table 1). Next, Caco-2 cells were treated with increasing concentrations of 5FU (6.25-200mM) for 24 hours to calculate the IC_{50} value of 5FU, the antineoplastic drug commonly used in colorectal chemotherapy. The IC_{50} value of 5FU was reported to be 20.07 µM (Table 1).

Table 1. IC_{50} of BBR and 5FU in Caco-2 cells for a 24 hour-period

Groups	IC_{50}	95% Confidence Interval (Min-Max)	r^2
BBR	281.4 μM	234.8-337.0	0.924
5FU	20.07 mM	15.94-25.03	0.937

Abbreviation: IC_{50}: Half-maximal inhibitory concentration, BBR: Berberin, 5FU: 5-fluorouracil

After demonstrating the cytotoxic effect of BBR on Caco-2 cells, we investigated whether BBR could further enhance the antiproliferative effect of 5FU. To this end, we determined the IC_{50} and $IC_{50}/2$ concentrations of 5FU (approximately 20 and 10 mM) and the IC_{50}x2, IC_{50}, $IC_{50}/2$ concentrations of BBR (560, 280, and 140 μM). Caco-2 cells were treated with each combination for 24 hours and cell viability was determined by MTT. The potential of synergistic cytotoxic effect was evaluated using Compusyn software based on the *Chou-Talalay* method. The results are shown in Table 2. The CI value of all combination groups was less than 1. The CI values indicate that the 5FU treatment in combination with BBR has a greater anticancer efficacy potential than monotherapy. In particular, the combination group (5FU (20 mM) + BBR (280 μM)), which consisted of IC_{50} concentrations, had the lowest CI value (0.143 ±0.012), i.e. the highest synergistic cytotoxic effect, and these concentrations were used in the following studies.

Table 2. The combination index (CI) of combinations of BBR with 5FU in Caco-2 cells.

Combinations	Effect (Fa)*	CI value	Interaction
5FU (20 mM) + BBR (560 μM)	0.071 ±0.006	0.201 ±0.042	Synergism
5FU (20 mM) + BBR (280 μM)	0.072 ±0.002	0.143 ±0.012	Synergism
5FU (20 mM) + BBR (140 μM)	0.128 ±0.005	0.530 ±0.055	Synergism
5FU (10 mM) + BBR (560 μM)	0.086 ±0.001	0.271 ±0.010	Synergism
5FU (10 mM) + BBR (280 μM)	0.135 ±0.005	0.573 ±0.056	Synergism
5FU (10 mM) + BBR (140 μM)	0.182 ±0.003	0.945 ±0.049	Synergism

Data are presented as mean ± standard deviation of three separate experiments. *Fa (fraction affected) represents the inhibition rate of the drug on Caco-2 cells.
BBR: Berberine, 5FU: 5-fluorouracil, CI: combination index.

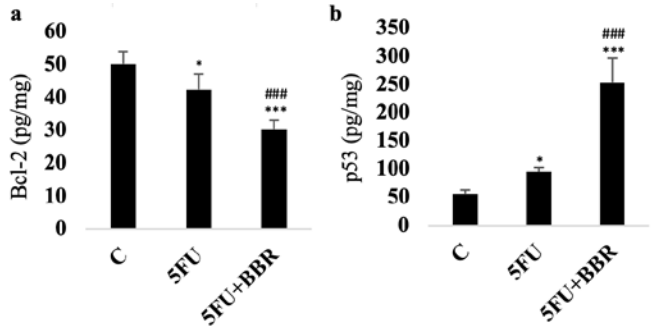

Figure 1. The effect of 5FU alone and 5FU+BBR treatment on anti-apoptotic protein Bcl-2 (a) and tumor suppressor protein p53 (b) in Caco-2 cells for 24 h. Results are mean ± standard deviation (n=3). *compared to C $p < 0.05$; * compared to C $p < 0.001$; #compared to 5FU $p < 0.05$; ###compared to 5FU $p < 0.001$.**

C: DMSO (0.5%), 5FU: 5-fluorouracil (in IC_{50} doses), BBR: Berberine (in IC_{50} doses).

Apoptosis levels (anti-apoptotic protein Bcl-2 and tumor suppressor protein p53) in Caco-2 cells treated with 5FU or BBR at IC_{50} and 24 hours per day were assessed by ELISA and are shown in Figure 1a-b. The means and standard deviations of Bcl-2 levels were calculated as 50.13±3.91, 42.43±4.82, and 30.21±2.83 pg/mg for 5FU and 5FU+BBR, respectively (Figure 1a). The mean values and standard deviations of p53 levels were calculated as 55.84±7.51, 95.46±7.17, and 252.96±43.70 pg/mg, respectively (Figure 1b). A significant decrease in Bcl-2 levels (0.712-fold compared to 5FU, $p<0.001$) and a significant increase in p53 levels (2.650-fold compared to 5FU, $p<0.001$) were observed in cells treated with 5FU+BBR, respectively (Figure 1a-b). L-6 and TNF-α, one of the important indicators of inflammatory response in Caco-2 cells treated with 5FU or BBR in IC_{50} and 24 hours with BBR, were evaluated by ELISA and shown in Figure 2a-b. The means and standard deviations of IL-6 levels were calculated as 1.23±0.09, 1.41±0.06, and 1.03±0.09 pg/mg in cells treated with 5FU and 5FU+BBR, respectively (Figure 2a). The means and standard deviations of TNF-α levels were calculated as 0.34 ± 0.04, 0.40 ± 0.05, and 0.20 ± 0.02 pg/mg (Figure 2b) in the control, 5FU and BBR treated cells, respectively. The IL-6 and TNF-α in the cells treated with 5FU are high compared to the control group ($p<0.05$), and significantly low in the cells treated with 5FU+BBR compared to 5FU (0.733-fold and 0.485-fold compared to 5FU, respectively, $p<0.001$) (Figure 2a-b). Furthermore, we investigated whether BBR in combination with 5FU has the ability to suppress MMP-9 in Caco-2 cells. The MMP-9 protein levels in the cells incubated with 5FU or BBR were calculated in IC_{50} concentrations of 745.36 ± 48.10, 727.49 ± 31.48, and 437.18 ± 25.65 pg/mg (Figure 2c) for the 5FU and 5FU+BBR groups, respectively. While no statistically significant difference was observed for treatment with 5FU ($p>0.05$), MMP-9 production was significantly inhibited by the combined theratment with 5FU+BBR (0.601-fold compared to 5FU, $p<0.001$) (Figure 2c).

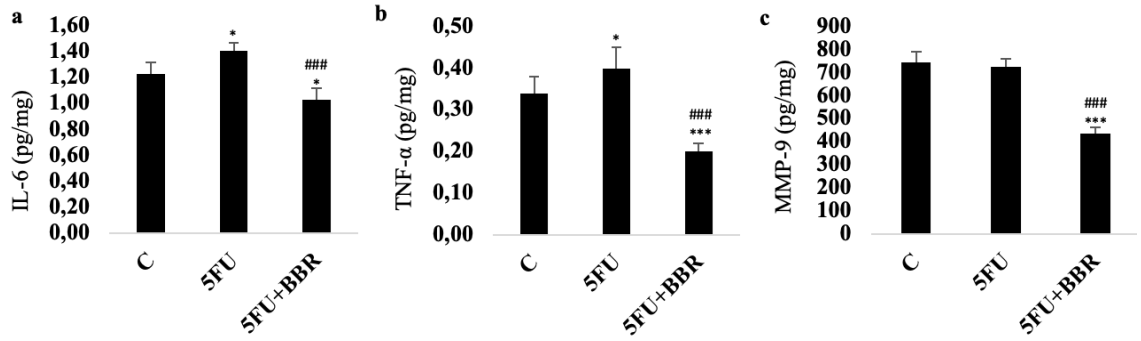

Figure 2. The effect of 5FU alone and 5FU+BBR treatment on IL-6 (a), TNF-α (b) and MMP-9 (c) in Caco-2 cells for 24 h. Results are mean ± standard deviation (n=3). *compared to C $p < 0.05$; * compared to C $p < 0.001$; #compared to 5FU $p < 0.05$; ###compared to 5FU $p < 0.001$.**

Abbreviations: C: DMSO (0.5%), 5FU: 5-fluorouracil (in IC_{50} doses), BBR: Berberine (in IC_{50} doses).

DISCUSSION

Colorectal cancer is one of the cancers with the highest prevalence and is a major cause of mortality[1]. Among the existing treatments, chemotherapy plays an important role. Targeted treatments that inhibit the angiogenic process or the epidermal growth factor receptor are currently used in combination with existing chemotherapeutic agents to treat colorectal cancer. However, despite developments in treatment, drug resistance and toxicity limit treatment[5,6]. Innovative pharmaceutical strategies are needed to increase treatment efficacy[8]. The incorporation of various phytochemicals characterized by potent anticancer activity has attracted the attention of many research groups in recent years, and promising results have been presented in the literature showing that combination treatment is more effective than monotherapy[35,36]. There are different opinions in the literature on

whether BBR, which is an effective and safe option for the treatment of colorectal cancer, can help to increase the therapeutic efficacy of 5FU, the currently widely used antineoplastic drug, and the mechanism underlying the potential synergistic effect has not been elucidated[10,27,32]. In this study, we performed colorimetric analysis of proteins related to cytotoxicity analysis, apoptosis, and inflammation, and investigated the potential role and mechanisms of combination therapy of 5FU and BBR in Caco-2 cells.

In our study, we first investigated the cytotoxic effect of BBR and 5FU in Caco-2 cells using the MTT assay. The cytotoxic evaluation revealed that both compounds had a dose-dependent antiproliferative effect on the Caco-2 cell line. The IC_{50} values for BBR and 5FU were 281.4 μM and 20.07 mM, respectively, over a 24-hour period. Although the IC_{50} values varied depending on the methods and cell lines used, the antiproliferative effect of BBR has been demonstrated in various colorectal cancer cell lines (HT-29, HCT116, SW480, SW620, LoVo, DLD-1, Caco-2) in agreement with our results [17,32,37].

We then investigated the potential synergistic cytotoxic effects in Caco-2 cells by forming combination groups of BBR (concentrations IC_{50}x2, IC_{50}, and IC_{50}/2) and 5FU (IC_{50}, IC_{50}/2) in unstable ratios. It was found that all combined treatments showed synergistic interactions (CI's <1) and higher cytotoxic effect compared with monotherapy. Our cytotoxicity data support the idea that BBR may help to improve the therapeutic efficacy of 5FU. The strongest synergistic effect (CI=0.143) was observed in the combination group, with both at IC_{50} values. 5FU and BBR were used in the calculation of IC_{50} values for the analysis of proteins associated with apoptosis and inflammation.

In recent years, BBR has been increasingly shown to enhance the therapeutic effect of 5FU and other antineoplastic drugs in various cancers, including colorectal cancer[25,28-32]. Recently, our study group reported that BBR can increase the therapeutic efficacy of sisplatin with apoptosis induction in the treatment of lung cancer[28]. Pandey et al.[29] reported that treatment of 5FU in combination with BBR has a significant antiproliferative effect with synergistic inhibition at survivin and STAT3 levels in AGS gastric cancer cells. Palmieri et al.[30] reported that the regulation of cell cycle and expression of epithelial-to-mesenchymal transition genes by BBR enhanced the anticancer effect of cisplatin and 5FU on human laryngeal cancer cell line. He et al.[31] reported that the combined treatment of BBR with 5FU and 2 other antineoplastic drugs (camptothecin and paclitaxel) at different doses had a synergistic cytotoxic effect on A549 non-small cell cancer cells and reduced multiple drug resistance (MDR) proteins. Cai et al.[32] reported that BBR alone had a cytotoxic effect on LoVo human colorectal adenocarcinoma cells and arrested the cell cycle (with the suppression of cyclin B1, CDC2, and CDC25C proteins) at an IC_{50} value (40.79 ± 4.11 μM). The same study team found that BBR (50 mg/kg) had a stronger antitumor effect in a human colorectal adenocarcinoma xenogreft compared to monotherapy (with 5FU or BBR). Yu et al.[25] reported that BBR increased chemosensitivity to irinotecan by suppressing the activation of antiapoptotic genes (c-IAP1, c-IAP2, survivin and Bcl-xL) and CPT-11-mediated nuclear factor-κB (NF-κB) in HCT-116 colon cancer cells. In contrast to the studies reporting synergistic effects, Bao et al.[27] showed the hormetic effect of BBR with antiproliferative effect at high doses (10-80 μM) while BBR increased the proliferation of various cancer cells at low doses (1.25-5 μM). They also reported that chemotherapeutic agents, including 5FU, camptothecin and paclitaxel at low doses can significantly reduce the anticancer effect. Therefore, BBR should be used with caution in the treatment of cancer. In a joint evaluation, it is important to determine the safe dose profile in the treatment of colorectal cancer and to find out the mechanism of potential synergistic toxicity of BBR and 5FU.

It is known that apoptosis is closely linked to inhibition of proliferation and that apoptosis is the key indicator in cancer treatment. The anti-apoptotic protein Bcl-2 and the tumor suppressor protein p53 play an important role in colorectal cancer and may serve as biomarkers and therapeutic targets[10,38,39]. In our study, BBR with 5FU upregulated the decreased Bcl-2 and increased p53 levels ($p<0.001$). The data on in vitro-induced apoptosis supported the synergistic inhibition of Caco-2 cell proliferation. In parallel with our results, BBR has been reported to prevent cancer cell proliferation by affecting cell cycle and autopagy in various cancer cells and stimulating cell apoptosis[40-42]. For example, BBR is thought to stimulate autophagy by stimulating the release of Beclin-1 from the Bcl-2/Beclin-1 complex in liver cancer cells[40]. In addition, BBR has been shown to stimulate apoptosis in several ways: Up-regulation of caspase-8 and caspase-9 in HL-60 human leukemia cells[41], elevation of cytochrome C levels in A431

squamous cells[42], and modulation of PI3K/AKT/mTOR signaling pathways in ATC anaplastic thyroid muscle carcinoma cells[43]. Apoptosis induction is also considered an important pathway for the potential synergistic effect between BBR and antineoplastic drugs used in various cancer treatments[10]. Doxorubicin, the only drug approved for the treatment of anaplastic thyroid cancer, has been reported to act synergistically with BBR to promote apoptosis and autophagation by ROS induced in these cancer cells[43]. In general, our results and the data in the literature support the idea that BBR, coupled with apoptosis induction, is a promising combination candidate for chemotherapy of colorectal cancer patients.

There is a significant association between inflammatory activation and imbalance of the gut microbiota and the occurrence and progression of colorectal cancer[44,45]. In colorectal cancer after primary tumor development, the role of inflammatory cytokines and tumor-infiltrating immune cells has been demonstrated[46]. Proinflammatory cytokines such as IL-6, IL-8, IL-1β, and TNF-α can influence microbiota-induced tumor formation by creating a microenvironment that promotes intestinal tumor development through multiple mechanisms including proliferation, angiogenesis, invasion, and metastasis[47,48]. Cytotoxic chemotherapeutic agents that trigger intestinal mucositis can lead to a deterioration of the mucosal barrier and cause damage to the gastrointestinal epithelium with activation of inflammatory coordination. This is unfavorable for the next steps in colorectal cancer treatment[7]. BBR may play a crucial role in colorectal cancer due to its anti-inflammatory and antioxidant effects[10]. In our study, inflammation by 5FU in Caco-2 cells was suppressed by BBR, with inhibitory effects on IL -6 and TNF-α ($p<0.001$). Consistent with our results, the study investigating the effect of BBR on 5FU-induced mucositis in cancer patients using an assessment model found a positive effect of BBR treatment on the gut microbiota and reported lower weight loss, lower diarrhea score, and longer column length. This study reported that the expression of IL-1β, IL-6 and TNF-α was suppressed and the intestinal mucosa in the ileum was damaged by BBR treatment[49]. In addition, treatment with BBR has been reported to reduce inflammatory factors such as IL-1β, TNF-a, C-C motif kemokine 1 (CCL1) and C-X-X motif ligand (CCL9) in intestinal tissue in the mouse colon cancer model (prepared with azoximeter and dextran sulfate sodium)[50]. In a similar study testing the effect of BBR on inflammatory diseases and cancer in a carcinogenesis mouse model associated with colitis, the application of BBR suppressed tumor formation, proinflammatory factors (IL-6, IL-1β, cycloxygenase-2, TNF-α) and the expression of cell proliferation indicator Ki67. In addition, expression levels of critical road proteins (P-Stat3 and P-JNK) in the inflammatory process and cell cycle regulatory molecules (β-catin, C-MYC and C-MYC and CYLind1) were reported to be lower[45]. In parallel with our results, findings from previous studies suggest that BBR may have beneficial effects on colorectal cancer by suppressing the inflammatory response and that BBR may be a good combination candidate.

During early metastasis of colorectal cancer, MMPs degrade the extracellular matrix, cause migration of cancer cells, and accelerate the progression of metastasis[51]. In our study, we observed that BBR in combination with 5FU significantly suppressed the migration and invasion of Caco-2 cells by significantly reducing MMP-9 protein levels compared to 5FU-treated cells. In previous studies consistent with our results, BBR inhibited the expression of proteins related to epithelial-gras transition and metastasis (such as MMP and E-cadherin), as well as cell migration and invasion in various cancers (colon[22], cervix[52], melanoma[53]). In a clinical trial, BBR was reported to be safe and effective in the prevention of colorectal adenocarcinoma[12]. Overall, MMP-9, which is known to be a good biomarker for early metastasis of colorectal cancer, can be suppressed by BBR alone or as part of chemotherapy.

Although BBR offers valuable and promising biological effects in colorectal cancer, the side effects of BBR should not be ignored[10]. On the other hand, the efficacy of BBR is limited as it has low bioavailability due to poor absorption from the intestine, low dissolution in water, and rapid metabolization. An oral bioavailability of 0.68% has been demonstrated in rats[54]. In recent years, there is increasing evidence that methods such as nanoparticle-based delivery systems used in the treatment of tumors could be an important potential strategy to improve the therapeutic effect of BBR[55]. We are also aware that our study has some limitations. The most commonly used combinations of chemotherapeutic agents, today such as FOLFOX (5FU, oxaliplatin and loekovorin), FOLFIRI (5FU,

irinotecan, loekovorin) or FOLFOXIR (5FU, oxaliplatin, irinotecan, loekovorin) could be evaluated for their potential synergistic effects by including different doses and time points[4]. In addition, potantial pathways of action such as cell cycle checkpoints, antioxidant defense system, and genotoxicity could be explored with further techniques. However, in vivo and clinical studies should be conducted to fill the gaps in the literature on how BBR alters the efficacy of antineoplastic drugs. Optimizing these approaches will pave the way for the use of BBR as part of chemotherapy.

In conclusion, our results demonstrate the enormous potential of BBR in combination with 5FU in the chemotherapy of colorectal cancer and may open a new perspective for cancer treatment. Existing studies and our results suggest that BBR may be a promising therapeutic candidate that could improve the efficacy of colorectal cancer treatment (monotherapy or combination therapy). However, due to the low water solubility and bioavailability of BBR, the development of nanotechnology formulations and their integration into the treatment may play a very important role. Evaluation of the in vitro results of this promising combination therapy with potential applications in animal models and clinical trials is worth investigating.

Author Contributions: Concept/Design: ŞÖ, MBK, SŞ; Data acquisition: MBK, SŞ; Data analysis and interpretation: SŞ, FÖKS; Drafting manuscript: ŞÖ, MBK, SŞ, FÖKS, SÇ; Critical revision of manuscript: ŞÖ, MBK, SÇ; Final approval and accountability: ŞÖ, MBK, SŞ, FÖKS, SÇ; Technical or material support: ŞÖ, SŞ, FÖKS, SÇ; Supervision: SÇ; Securing funding (if available): n/a.
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Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
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