Synergistic Effects of 5-Fluorouracil in Combination with Diosmetin in Colorectal Cancer Cells †

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Abstract: Colorectal cancer (CRC) is among the most commonly occurring cancers. The management of CRC includes laparoscopic surgery, radiotherapy, chemotherapies and neoadjuvant treatment. However, conventional chemotherapies have poor impact on combating CRC and are associated with severe toxic effects and high rates of relapse. Therefore, searching for a new combination regimen is a favorable consideration. The aim of this study was to elucidate the synergistic effect of 5-fluorouracil (5-FU) and diosmetin in an in vitro model on colorectal cancer cells. An MTT assay was conducted on HCT-116 cancer cells and they were treated with a concentration gradient of 5-FU and diosmetin individually and in combination. The combination index (CI) and dose reduction index (DRI) were calculated using CompuSyn software. Isobologram analysis and synergism determination were performed using the Combenefit software tool and the synergy score was calculated using the SynergyFinder 2.0 software tool. The apoptotic features of the cells were determined via an AO/PI double staining assay and an annexin V assay using a fluorescent microscope and the flow cytometry technique, respectively. The findings showed that the DRI of 5-FU was three-fold lower in the combination with a CI value of less than one, which indicates that there was a synergistic effect. The AO/PI microscopic results revealed signs of apoptosis and dead cells after 72 h of treatment. Flow cytometry analysis confirmed that the apoptotic effect of the combination was more prominent compared to 5-FU alone. The findings of this study offer a potential strategy for reducing the cytotoxicity and enhancing the efficacy of 5-FU on colorectal cancer cells through a synergistic study model.

Keywords: colorectal cancer; synergism; 5-fluorouracil; diosmetin; combination index; dose reduction index

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second in terms of mortality. According to GLOBOCAN, an estimation of 1,148,515 new CRC cases and 576,858 colorectal deaths were detected in 2020 [1]. Although significant therapeutic improvements have been observed for CRC, the main concern still revolves around relapse and metastasis, which leads to poorer prognosis. Therefore, searching for a novel therapeutic approach is necessary to overcome CRC health complications [2]. One intriguing strategy is the synergistic combination of current chemotherapeutic drugs with natural and safe bioactive compounds. A standard chemotherapeutic drug against CRC is 5-Fluorouracil (5-FU), which acts by causing damage to the DNA [3]. However, significant side effects are associated with this drug, which inspired several researchers to combine 5-FU with bioactive phytoconstituents, suggesting that this therapeutic strategy may reduce the toxic side effects of 5-FU and enhance its efficacy [4]. Diosmetin is a flavonoid compound found in citrus and other medicinal plants. Numerous studies
have demonstrated that this compound suppresses cancer cell proliferation, including hepatocarcinoma, leukemia, breast cancer, lung, CRC and prostate cancer [5–10]. Therefore, in this study, we investigated the synergistic effect of 5-FU in combination with diosmetin against the HCT-116 colorectal cancer cell line.

2. Methodology

2.1. Cell Line and Culture Condition

The colon cancer cell (HCT-116) was purchased from the American Type Culture Collection (ATCC, VA, USA). The cells were grown in McCoy’s 5a media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were incubated under ideal conditions (5% CO₂ and 95%, humidity at 37°C).

2.2. MTT Assay to Determine Cell Viability Percentage (%)

An MTT assay was conducted according to a method described previously [11]. Cells were treated with a serial range of concentrations (100 to 0.78 µg/mL) of the single drugs (diosmetin or 5-FU) followed by incubation for 72 h. Formazan crystals were dissolved with DMSO and the optimal density was recorded using a micro plate reader at 570 nm. The percentage of cell viability was estimated in comparison to the untreated control cells [11]. Upon IC₅₀ determination from a single drug, cells were exposed to a combination treatment that included diosmetin and 5-FU at a fixed ratio of doses using higher and lower doses than the individual IC₅₀ [9].

2.3. AO/PI Double Staining Assay

A microscopic viability assessment of the HCT-116 cells after treatment with diosmetin, 5-FU and a combination was conducted using acridine orange and propidium iodide (AO/PI) fluorescent dyes. In brief, cells were treated with the IC₅₀ doses of each individual drug and in combination for 72 h. Cell pellets was stained with 10 µL of AO/PI. A fluorescent inverted microscope was used to detect morphological changes of the cells. The resulting green, orange and red colors represent viable, early apoptotic and necrotic cells, respectively [9].

2.4. Annexin V/PI Assay for the Detection of Cell Apoptosis

This assay was conducted to identify the apoptotic effect of the combination compared to individual drug treatments. In brief, cells were treated with the IC₅₀ doses of each individual drug and in combination for 72 h. Cell pellets were stained with PI and FITC-annexin V for 15 min, and introduced to the FACSCaliber flow cytometer instrument to assess apoptosis [9].

2.5. Statistical Analysis

The combination index (CI) and dose reduction index (DRI) were calculated using the Chou–Talalay equation [12] and CompuSyn software. Isobologram analysis and synergism determination were carried out using Combenefit software v2.021. The synergy score was calculated using SynergyFinder 2.0 software.

3. Results

3.1. MTT

The IC₅₀ of diosmetin and 5-FU were 4.16 ± 1.3 and 0.83 ± 0.0 µg/mL, respectively. The treatment of cells with both drugs induced growth inhibition in a dose-dependent manner. Upon the IC₅₀ determination, a combination therapy was designed based on a fixed ratio (1:5). The doses covered the IC₅₀ values in addition to higher and lower doses than IC₅₀. Cells were treated with these doses of individual drugs and in combination for 72 h. The combination of the two drugs inhibited cell growth in a dose-dependent manner with an IC₅₀ of 5-FU (0.27 µg/mL) lower than the IC₅₀ of 5-FU as a single treatment.
CompuSyn analysis represented a range of CI values for each combined dose. The CI value of 0.66 (<1) was detected based on the Chou–Talalay method [12]. The DRI of 5-FU was estimated as 3.0, which indicates a three-fold reduction. Combenefit analysis (Figure 1) confirmed the CompuSyn findings by detecting the significant synergistic effect at the dose of 0.15 to 0.62 µg/mL of 5-FU and 0.78 to 6.25 µg/mL of diosmetin in combination. To confirm the synergism effect, the synergy score as the average excess response to drug exposure was calculated using SynergyFinder 2.0, and, as a result, the synergy score obtained was 17.051 ± 1.67 (>10 is synergistic), which indicates a 17.051% response.

Figure 1. Combenefit analysis of diosmetin and 5-FU combination. Data were obtained from three individual experiments. Data are presented as mean ± SD, * p < 0.05, ** p < 0.001, *** p < 0.0001 indicate significant differences compared to monotherapy.

3.2. AO/PI Double Staining Assay

The detection of apoptosis using the AO/PI staining technique was considered in this study. As shown in Figure 2, a notable difference can be observed in apoptosis induction between the control group and treated groups with 5-FU, diosmetin and their combination. The application of 5-FU showed higher necrotic cells while diosmetin treatment resulted in more apoptotic cells (blebbing and chromatin condensation). Treatment with the combination of the two drugs showed apoptotic cells with less necrotic cells as compared to 5-FU-treated cells.
3.3. Annexin V-FITC Assay

The induction of cellular apoptosis by the individual drugs and in combination was measured using an annexin V-FITC assay. The results showed that 5-FU has a greater percentage of necrotic cells (39.9%) while diosmetin and the combination treatment demonstrated a lower percentage of necrosis (15.2% and 12.1%, respectively), with a higher percentage of apoptotic cells (41.9%) in the combination treatment as compared to 37.3% for the 5-FU treatment (Figure 3).
4. Discussion

In the current study, the synergistic effect of 5-FU and diosmetin in combination was assessed and the growth inhibitory effect was analyzed using Chou–Talalay method [12]. The HCT-116 cells treated with the combination of 5-FU and diosmetin showed a CI value of less than 1, which indicates a synergistic effect. In addition, the IC\textsubscript{50} of 5-FU in the combination (0.27 $\mu$g/mL) demonstrated a three-fold reduction as compared to the IC\textsubscript{50} of 5-FU (0.83 $\mu$g/mL), which indicates less adverse effects compared to 5-FU as a chemotherapeutic agent.

To further reiterate the apoptotic effect of the combined drugs, an AO/PI double staining assay was conducted to characterize the viable and dead cells based on the morphological changes using a fluorescent microscope. AO is able to penetrate the membrane of viable cells at an early apoptotic stage with fragmented DNA, whereas PI only stains dead cells [13]. The results of this assay showed the intact nuclei (green cells) in the control group, and membrane blebbing and chromatin condensation in the cells treated with diosmetin and the combination as a sign of apoptosis. This observation is in consonance with previously reported data on the effect of diosmetin on colorectal cancer cells [9]. Late apoptosis and necrotic cells (red cells) were mostly detected in 5-FU-treated cells, though some were in the combination-treated cells.

Further apoptotic assessment was conducted using annexin V-FITC-PI staining. Phosphatidylserine (PS) translocation to the outer membrane space is a typical biomarker of apoptosis. In this study, this biomarker was detected during flow cytometry using FITC and PI dyes [13]. The highest percentage of apoptosis was detected in the combination-treated cells with 41.9% as compared to 5-FU and diosmetin with 37.3% and 41.5%, respectively. In addition to apoptosis, flow cytometry detected the percentage of necrotic cells, whereby cells were annexin V negative and PI positive. In this study, the percentage of necrotic cells decreased in the combination group in comparison to the 5-FU treatment group. Over-
all, the results suggest that combination therapies are more potent than 5-FU alone, and that diosmetin exerts a synergistic effect when combined with 5-FU. In addition, the cytotoxicity effect of combination against HCT-116 cells was apparent through the induction of apoptosis.

5. Conclusions

Overall, this study has provided evidence that 5-FU and diosmetin exert a synergistic effect against HCT-116 cells via apoptosis induction. However, further assessments are required to detect the molecular mechanism of the combination therapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ECB2021-10276/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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