ECB 2021

sciforum

Proceedings

Synergistic effects of 5-fluorouracil in combination with diosmetin in colorectal cancer cells †

Sareh Kamran, Ajantha Sinniah, Mohammed Abdullah Alshawsh*

Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

* Correspondence: alshaweshmam@um.edu.my

† Presented at the 1st International Electronic Conference on Biomedicine, 1–26 June 2021; Available online: https://ecb2021.sciforum.net/.

Published: 31 May 2021

Abstract: Colorectal cancer (CRC) is among the most common occurring cancer. The management of CRC includes laparoscopic surgery, radiotherapy, chemotherapies and neoadjuvant. However, the conventional chemotherapies have poor impact on combating CRC and are associated with severe toxic effects and high 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. MTT assay was conducted on HCT-116 cancer cells and treated with concentration gradient of 5-FU and diosmetin individually and in combination. Combination index (CI) and dose reduction index (DRI) were calculated using CompuSyn software. Isobologram analysis and synergism determination was done via Combenefit software tool and synergy score was calculated using SynergyFinder 2.0 software tool. Apoptotic features of cells were determined via AO/PI double staining assay and Annexin V assay using fluorescent microscope and flow cytometry technique, respectively. Findings showed that the DRI of 5-FU was three-fold lower in the combination with CI value less than one, which indicates a synergistic effect. AO/PI microscopic results revealed signs of apoptosis and dead cells after 72 h of treatment. Flow cytometry analysis confirmed the apoptotic effect of combination was more prominent as compared to 5-FU alone. The findings of this study offered a potential strategy to reduce the cytotoxicity and enhance 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 are observed in CRC, yet the main concern still revolves around relapse and metastasis, that 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. 5-Fluorouracil (5-FU) is a standard chemotherapeutic drug against CRC, which acts by causing damage to DNA [3]. However, significant side effects are associated with this drug, which inspired several researchers to combine 5-FU with bioactive phytoconstituents, suggesting this therapeutic strategy may reduce the toxic side effects of 5-

FU and enhances its efficacy [4]. Diosmetin is a flavonoid compound found in citrus and other medicinal plants. Numerous studies have demonstrated that this compound suppresses cancer cells proliferation such as 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 HCT-116 colorectal cancer cell line.

2. Methodology

2.1. Cell line and culture condition

The colon cancer cell (HCT-116) was purchased from American Type Culture Collection (ATCC, 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% CO_2 and 95% Humidity at 37 °C).

2.2. MTT assay to determine cell viability percentage (%)

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 hours. Formazan crystals were dissolved with DMSO and 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 IC_{50} determination from single drug, cells were exposed to a combination treatment including diosmetin and 5-FU at a fixed ratio of doses using higher and lower doses than the individual IC_{50} [12].

2.3. AO/PI double staining assay

Microscopic viability assessment of HCT-116 cells after treatment with diosmetin, 5-FU and combination was detected using acridine orange and propidium iodide (AO/PI) fluorescent dyes. In brief, cells were treated with the IC_{50} doses of each individual drug and in combination for 72h. Cell pellets was stained with 10 µL of AO/PI. Fluorescent inverted microscope was used to detect morphological changes of the cells. The resulted green, orange and red colors represents viable, early apoptotic and necrotic cells, respectively [13].

2.4. Annexin V/PI assay for the detection of cell apoptosis

This assay was conducted to identify the apoptotic effect of the combination as compared to individual drug treatments. In brief, cells were treated with the IC_{50} doses of each individual drug and in combination for 72h. Cell pellets were stained with PI and FITC-Annexin V for 15 minutes, and introduced to the FACS Caliber flow cytometer instrument to assess apoptosis [13].

2.5. Statistical analysis

Combination index (CI) and dose reduction index (DRI) were calculated using Chou Talalay equation [12] and CompuSyn software. Isobologram analysis and synergism determination were carried out via Combenefit software. Synergy score was calculated using SynergyFinder 2.0 software.

3. Results

3.1. MTT

The IC_{50} of diosmetin and 5-FU were 4.16 ± 1.3 and 0.83 ± 0.0 µg/mL, respectively. Treatment of cells with both drugs, induced growth inhibition in a dose dependent manner. Upon the IC_{50} determination, a combination therapy was designed based on a fixed ratio (1:5). The doses covered the IC_{50} values in addition to higher and lower doses than IC_{50}. Cells were treated with these doses of individual drugs and in combination for 72h. The combination of the two drugs inhibited cell growth

in a dose dependent manner with an IC_{50} of 5-FU (0.27 µg/mL) lower than the IC_{50} 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 Chou Talalay method [12]. DRI of 5-FU was estimated as 3.0, which indicates 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, synergy score as the average excess response to drug exposure was calculated using SynergyFinder 2.0, as a result the synergy score obtained as 17.051 ± 1.67 (>10 is synergistic), which indicates 17.051% response.

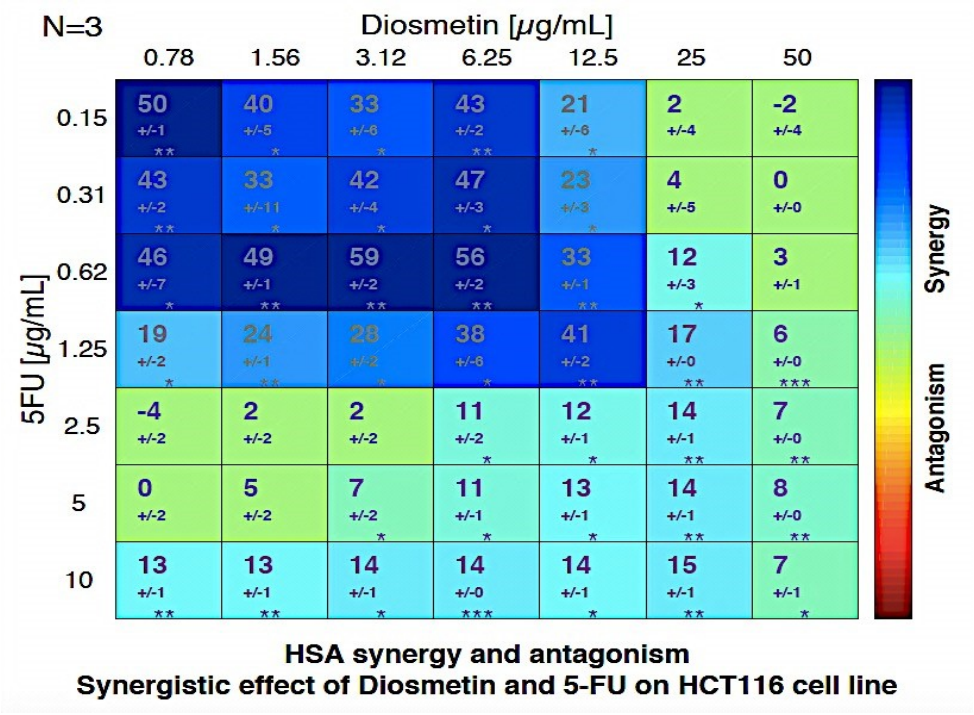

Figure 1. Combenefit analysis of diosmetin and 5-FU combination. Data were obtained from three individual experiments.

3.2. AO/PI double staining assay

Detection of apoptosis via AO/PI staining technique was considered in this study. As shown in Figure 2, notable difference is observed in apoptosis induction between the control group and treated groups with 5-FU, diosmetin and combination. 5-FU showed higher necrotic cells while diosmetin treatment resulted in more apoptotic cells (blebbing & chromatin condensation). Treatment with the combination of the two drugs showed apoptotic cells with less necrotic cells as compared to 5-FU treated cells.

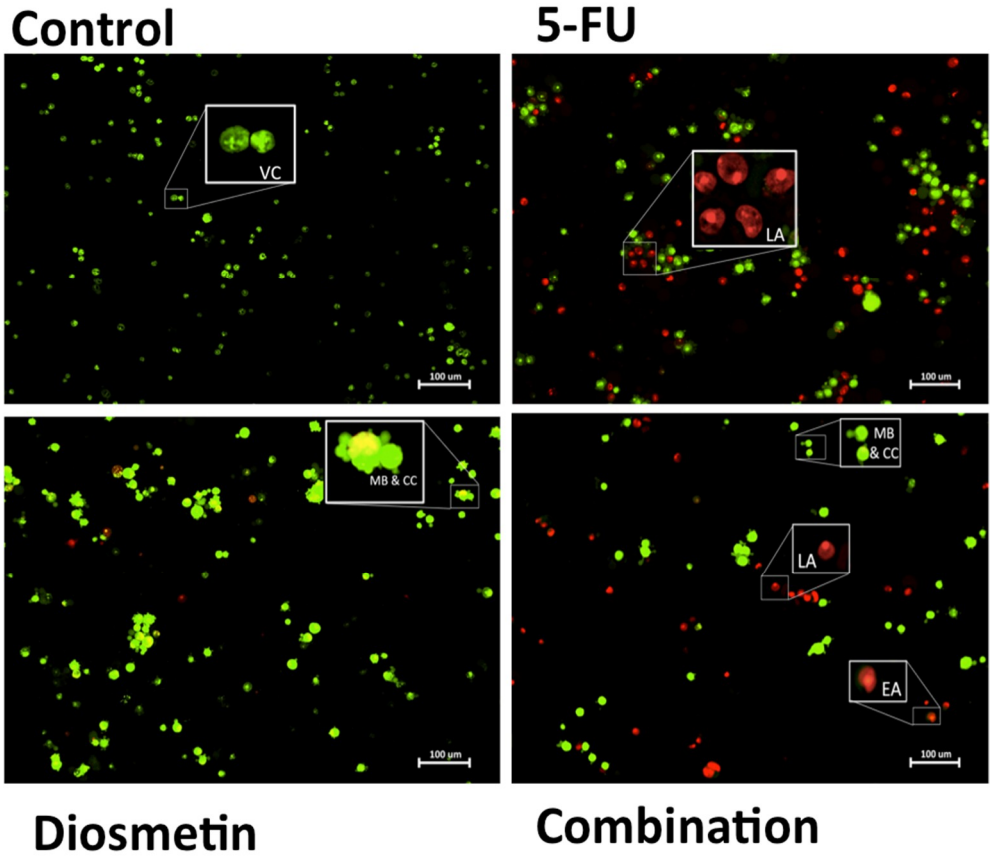

Figure 2. AO/PI staining of HCT-116 cells after 72h treatment with the IC_{50} of 5-FU, diosmetin and combination as compared to untreated control cells. VC: viable cells, LA: late apoptosis, MB: membrane blebbing, CC: chromatin condensation, EA: early apoptosis and N: necrosis. 10x magnification.

3.3. Annexin V-FITC assay

Induction of cellular apoptosis by individual drugs and in combination was measured via annexin V-FITC assay. The results showed that 5-FU has greater percentage of necrotic cells (39.9%) while diosmetin and combination treatment demonstrated less 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% of 5-FU treatment (Figure 3).

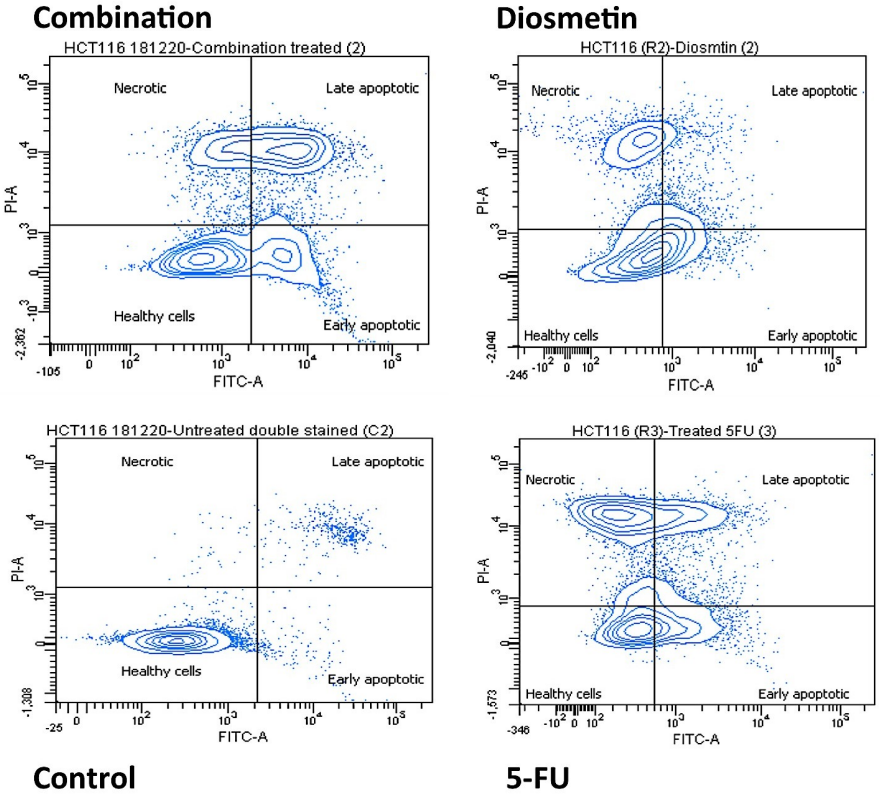

Figure 3. Flowcytometry graph of annexin V-FITC analysis in HCT116 cells. Cells were treated with the IC_{50} doses of the individual drugs and in combination.

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 by Chou Talalay method [12]. The HCT-116 cells treated with the combination of 5-FU and diosmetin showed CI value less than 1, which indicates a synergistic effect. In addition, the IC_{50} of 5-FU in the combination (0.27 µg/mL) demonstrated three-fold reduction as compared to the IC_{50} of 5-FU (0.83 µg/mL), which indicates less adverse effects compare to 5-FU as a chemotherapeutic agent.

To further reiterate the apoptotic effect of the combined drugs, AO/PI double staining assay was conducted to characterize viable and dead cells based on the morphological changes using fluorescent microscope. AO is able to penetrate membrane of viable cells at early apoptotic stage with fragmented DNA, whereas PI only stains dead cells [14]. The results of this assay showed intact nuclei (green cells) in control group, and membrane blebbing and chromatin condensation in treated cells with diosmetin and combination as a sign of apoptosis. This observation is in consonance with a previously reported data on the effect of diosmetin on colorectal cancer cell [13]. Late apoptosis and necrotic cells (red cells) were mostly detected in 5-FU treated cells and some in combination treated cells.

Further apoptotic assessment was done using Annexin V-FITC-PI stain. Phosphatidylserine (PS) translocation to the outer membrane space is a typical biomarker of apoptosis. In this study, this biomarker was detected by flow cytometry using FITC and PI dyes [14]. The highest percentage of apoptosis was detected in 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 combination group in comparison to 5-FU treatment group. Overall, the

results suggest that combination therapy are more potent than 5-FU, and diosmetin exerts a synergistic effect when combined with 5-FU. In addition, the cytotoxicity effect of combination against HCT-116 cells was through the induction of apoptosis.

5. Conclusion

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.

Funding: This study was funded by FRGS research grant from the Ministry of Higher Education, Malaysia ((FP103-2019A) Ref: FRGS/1/2019/SKK10/UM/02/3).

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

References

[1] Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.*, **2021**. https://doi.org/10.3322/caac.21660.

[2] Ciardiello, D.; Vitiello, P. P.; Cardone, C.; Martini, G.; Troiani, T.; Martinelli, E.; Ciardiello, F. Immunotherapy of Colorectal Cancer: Challenges for Therapeutic Efficacy. *Cancer Treatment Reviews*. 2019. https://doi.org/10.1016/j.ctrv.2019.04.003.

[3] He, L.; Zhu, H.; Zhou, S.; Wu, T.; Wu, H.; Yang, H.; Mao, H.; SekharKathera, C.; Janardhan, A.; Edick, A. M.; et al. Wnt Pathway Is Involved in 5-FU Drug Resistance of Colorectal Cancer Cells. *Exp. Mol. Med.*, **2018**. https://doi.org/10.1038/s12276-018-0128-8.

[4] Ghosh, S.; Pal, A.; Ray, M. Methylglyoxal in Combination with 5-Fluorouracil Elicits Improved Chemosensitivity in Breast Cancer through Apoptosis and Cell Cycle Inhibition. *Biomed. Pharmacother.*, **2019**. https://doi.org/10.1016/j.biopha.2019.108855.

[5] AlAjmi, M. F.; Rehman, M. T.; Hussain, A.; Rather, G. M. Pharmacoinformatics Approach for the Identification of Polo-like Kinase-1 Inhibitors from Natural Sources as Anti-Cancer Agents. *Int. J. Biol. Macromol.*, **2018**. https://doi.org/10.1016/j.ijbiomac.2018.05.023.

[6] Androutsopoulos, V. P.; Mahale, S.; Arroo, R. R. J.; Potter, G. Anticancer Effects of the Flavonoid Diosmetin on Cell Cycle Progression and Proliferation of MDA-MB 468 Breast Cancer Cells Due to CYP1 Activation. *Oncol. Rep.*, **2009**. https://doi.org/10.3892/or_00000384.

[7] Oak, C.; Khalifa, A. O.; Isali, I.; Bhaskaran, N.; Walker, E.; Shukla, S. Diosmetin Suppresses Human Prostate Cancer Cell Proliferation through the Induction of Apoptosis and Cell Cycle Arrest. *Int. J. Oncol.*, **2018**. https://doi.org/10.3892/ijo.2018.4407.

[8] Xu, Z.; Yan, Y.; Xiao, L.; Dai, S.; Zeng, S.; Qian, L.; Wang, L.; Yang, X.; Xiao, Y.; Gong, Z. Radiosensitizing Effect of Diosmetin on Radioresistant Lung Cancer Cells via Akt Signaling Pathway. *PLoS One*, **2017**. https://doi.org/10.1371/journal.pone.0175977.

[9] Koosha, S.; Mohamed, Z.; Sinniah, A.; Alshawsh, M. A. Investigation into the Molecular Mechanisms Underlying the Anti-Proliferative and Anti-Tumorigenesis Activities of Diosmetin against HCT-116 Human Colorectal Cancer. *Sci. Rep.*, **2019**, *9* (1), 1–17. https://doi.org/10.1038/s41598-019-41685-1.

[10] Roma, A.; Rota, S. G.; Spagnuolo, P. A. Diosmetin Induces Apoptosis of Acute Myeloid Leukemia Cells. *Mol. Pharm.*, **2018**. https://doi.org/10.1021/acs.molpharmaceut.7b01151.

[11] Yang, T.; Aimaiti, M.; Su, D.; Miao, W.; Zhou, B.; Maimaitiyiming, D.; Yusup, A.; Upur, H.; Aikemu, A. Enhanced Efficacy with Reduced Toxicity of Chemotherapy Drug 5-Fluorouracil by Synergistic Treatment with Abnormal Savda Munziq from Uyghur Medicine. *BMC Complement. Altern. Med.*, **2017**. https://doi.org/10.1186/s12906-017-1685-4.

[12] Chou, T. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. **2010**, *70* (2), 440–447. https://doi.org/10.1158/0008-5472.CAN-09-1947.

[13] Koosha, S.; Mohamed, Z.; Sinniah, A.; Alshawsh, M. A. Investigation into the Molecular Mechanisms Underlying the Anti-Proliferative and Anti-Tumorigenesis Activities of Diosmetin against HCT-116 Human Colorectal Cancer. *Sci. Rep.*, **2019**, *9* (1), 1–17. https://doi.org/10.1038/s41598-019-41685-1.

[14] Bagheri, E.; Hajiaghaalipour, F.; Nyamathulla, S.; Salehen, N. The Apoptotic Effects of Brucea Javanica Fruit Extract against HT29 Cells Associated with P53 Upregulation and Inhibition of NF-KB Translocation. *Drug Des. Devel. Ther.*, **2018**. https://doi.org/10.2147/DDDT.S155115.