Diosmetin Exerts Synergistic Effects in Combination with 5-Fluorouracil

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Submitted by: Mohammed Alshawsh

Definition

5-Fluorouracil (5-FU) is a chemotherapeutic medication commonly used to treat colorectal cancer (CRC); however, the drug-associated adverse effects and toxicity have greatly affected its clinical use. Diosmetin, a natural flavonoid, has been shown to inhibit the proliferation of many cancer cells, including CRC cells. The findings suggest that 5-FU/diosmetin combination exhibits synergistic effect against HCT116 cancer cells, and potentially reduces the unfavorable adverse effect of 5-FU while enhancing the anticancer efficacy by inducing apoptosis and interrupting mitosis.

1. Introduction

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and the second most deadly cancer. Although there have been substantial advances in the chemotherapeutic agents against CRC, severe adverse effects and toxicity are the major clinical problems. Hence, exploring other potential therapeutic strategies to combat CRC is crucial [1]. The synergistic effect of existing chemotherapeutic drugs in combination with natural and safe bioactive agents is an effective approach that has been considered in previous studies [2][3][4][5][6]. There are several chemotherapeutic drugs such as doxorubicin, cisplatin, methotrexate, 5-fluorouracil (5-FU), and paclitaxel that are used in combination therapy [7]. The current combination therapies include FOLFOX regimen (folinic acid + 5-FU + oxaliplatin), FOLFIRI regimen (folinic acid + 5-FU + irinotecan), XELOX or CAPOX regimen (capecitabine and oxaliplatin), and CAPIRI regimen (capecitabine + irinotecan) [8]. Although the numbers of new chemotherapeutic agents have increased, usage of 5-FU combined therapy remains a preferred control measurement for the treatment of CRC. However, severe cytotoxic effects and other toxicities are still the main concern for conventional chemotherapy combinations [9].

2. Effect of Combination on Cell Proliferation

Both monotherapy and combination therapy inhibited HCT116 cell growth in a dose-dependent manner (Figure 1). Based on the IC50 findings of monotherapy, a fixed constant ratio of 5-FU and diosmetin (1:5) was used to perform combination therapy with a combination regimen covering the IC50 values, as well as higher and lower concentrations. Figure 1 shows that the combination of diosmetin and 5-FU inhibited HCT116 cell growth more potently than monotherapy, and reduced the IC50 of 5-FU and diosmetin to 0.27 ± 1.1 and 1.38 ± 0.8 μg/mL, respectively.

Figure 1. Cell growth inhibitory effect of diosmetin (D) and 5-FU as monotherapy and combination therapy in HCT116 cells. Data were expressed as mean ± standard deviation (SD). * p < 0.05 and ** p < 0.01 indicate significant differences compared to the untreated control (0).

3. Synergistic Effect of Combination

The CI value for combination against HCT116 cells was calculated according to the Chou Talalay equation...
The mean CI value was 0.66 ± 0.4, which indicates a synergistic effect of combination therapy on HCT116 cells. Moreover, the mean DRI of 5-FU in the combination therapy was 3.0 ± 1.9, which suggests a three-fold dosage reduction compared to monotherapy. Figure 2 shows the CI plot of combination in HCT116 cells generated by CompuSyn software (version 1.0; ComboSyn, Paramus, NJ, USA), which plotted CI against the inhibitory effect. The plot revealed a synergistic pattern (CI < 1).

![Combination index plot](image)

**Figure 2.** Combination index plot (Fa-CI plot) of interaction between 5-FU and diosmetin (D) in HCT116 cells generated by CompuSyn software. Fa: inhibitory effect, CI: combination index. Fa of 0.5 represents 50% growth inhibition.

### 4. The Synergistic Interactions in Combination Therapy

Combination therapy is based on the positive effects of pharmacodynamic interactions (synergistic or additive) between two or more drugs, with synergistic interactions resulting in more effective treatments. In combination therapy, both compounds are given at lower doses and interact with multiple molecular pathways; therefore, combined treatments based on compounds that exhibit a synergistic or additive effect usually have less toxicity than monotherapy. Combination therapy has shown various advantages over monotherapy, including decreasing drug concentration and toxicity, enhancing the efficacy, targeting several molecular pathways, and sensitizing cells to the treatment.

Conventional chemotherapy medications are usually used in combination for the treatment of different types of cancer including CRC, this combination therapy is associated with serious adverse effects. Therefore, introducing bioactive anticancer natural compounds in combination therapy may promote chemotherapy efficacy and reduce the toxic adverse effects. In addition, natural bioactive compounds have more structural diversity, bioactivity, and complexity than synthetic drugs, and can inhibit some targets previously thought to be undruggable. They also inherently target biologically relevant pathways, because most natural bioactive compounds are secondary metabolites or signaling molecules. In addition, there is a limited overlap between the molecular signaling targeted by natural products and those targeted by synthetic drugs. This property not only indicates the potential for novel therapeutic targets for CRC but can also assist to lower the cost of developing new agents by utilizing compounds that already exist in nature and providing another option for combination therapy. Furthermore, patients receiving FOLFOX regimen, which is the most regularly used chemotherapy regimen for the treatment of CRC, usually suffer from different gastrointestinal, neurological, respiratory and skin adverse effects, including hair loss. Another example of conventional chemotherapy combination is the DCF (docetaxel + cisplatin + 5-FU) regimen, which is linked to stomatitis, diarrhoea, nausea, vomiting, neuropathy and associated with high toxicity.
5. The Synergistic Interaction between 5-FU and Diosmetin in HCT116 and HT29 Colorectal Cancer Cells

Since the proliferative ability of cancer cells is crucial for the tumor’s growth [14], the findings revealed that different concentrations of 5-FU and diosmetin dose-dependently inhibited the proliferation of HCT116 and HT29 cells. Moreover, based on interaction analysis using different software, combination therapy showed a synergistic effect in HCT116 cells with CI value less than one and synergy score more than 17. The IC_{50} of 5-FU reduced by 3-folds from 0.83 µg/ml to 0.27 µg/ml, which is favorable to reduce the sever toxicity and adverse effects associated with 5-FU chemotherapy. Other researchers also demonstrated that diosmetin can interact synergistically with anticancer drugs in other cancer cells, for example, diosmetin combined with paclitaxel synergistically induced apoptosis in non-small cell lung cancer cells via Nrf2 inhibition through disruption of PI3K/Akt/GSK-3β pathway [13]. Additionally, diosmetin generated a synergistic cytotoxic effect in HepG2 cells via cytochrome P450, family 1 (CYP1)-catalyzed metabolism, activation of c-Jun N-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK), and p53/p21 overexpression [16].

On the other hand, the combination therapy induced an additive effect in HT29 cells with CI value equal to one and synergy score of −3.824. Although both cell lines are colorectal cancer cells, HT 29 and HCT-116 represent different extents of mutation and differentiation. HT29 is a p53 mutated type and has an intermediate capacity to differentiate into enterocytes and mucin-expressing lineages, while HCT116 is known to be a highly aggressive wild type cell line that shows no ability to differentiate. Different interactions effects of 5-FU and diosmetin combination on these two cancer cell lines could be attributed to the differences in mutation and differentiation, however the additive effect in HT29 cells still contributes to the beneficial effects of this combination. Another possible reason for the different interaction effect in HCT116 and HT29 cells could be due to the differences in their genetic profiles. Sensitive p53 wild type cancer cells such as HCT116 are usually targeted via p53-mediated apoptosis while mutated or null p53 cells such as HT29 cells can be inhibited by drugs that induce p53-independent cell death pathways [17].

Imbalance between proliferation and apoptosis is a major element in the initiation of cancer. Apoptosis serves as a key function in correcting normal tissue stability [18]. Therefore, therapy options that target apoptosis may be effective in preventing the progression of CRC. Apoptosis is characterized by cell shrinkage, chromatin, nuclear condensation, and plasma membrane blebbing [19]. AO/PI staining and Annexin V-FITC were conducted to detect whether the suppression of HCT116 cells proliferation induced by combination therapy was associated with apoptosis. The treated cells exhibited apoptotic characteristics, including membrane blebbing in early apoptosis and chromatin condensation in late apoptosis. Before the cellular membrane disintegrates during early apoptosis, phospholipid asymmetry occurs [20][21]. Phosphatidylserine (PS) translocates to the outer plasma membrane, where it is exposed to the exterior surface. As a result, PS translocation can be used to investigate apoptosis. Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for PS, and it is frequently used in conjunction with PI (fluorescent dye) to identify apoptotic and necrotic cells [14]. To further quantify the apoptotic HCT116 cells following the treatment with monotherapy and combination, cells were exposed to Annexin V/PI staining and subjected to flow cytometry. Combination therapy significantly increased apoptotic cells to 45%, compared with 5-FU-treated cells, which showed only 24.6% of apoptosis. There were more necrotic cells (34.4%) in HCT116 cells treated with 5-FU than combination-treated cells (19.1%). It has been reported that chemotherapeutic drugs not only trigger apoptosis, but also other types of cell suicide, such as necrosis, which triggers further inflammation. Thus, it is not a preferred pathway for cancer treatment [22]. Therefore, the combination of 5-FU and diosmetin has the advantage to act through activating the apoptosis pathway with less impact on the necrosis pathway compared with 5-FU.

6. Combination Therapy in Anti-Cancer Management

Several dysregulated signaling pathways have been linked to cancer development. Conventional
chemotherapy agents have toxicity and severe adverse effects. Therefore, finding new multi-targeted treatment to reduce cancer’s dysregulated signaling is critical [23]. Diosmetin was reported to have a potential effect on signaling pathways involved in colorectal cancer. These pathways include apoptosis, TGF-β/BMP, NF-κB [24], PI3K/AKT [25], and Notch signaling pathways [26]. A combination of diosmetin with chemotherapeutic drug (5-FU) could target multiple signaling pathways and produce a higher response rate against colorectal cancer. Combination therapy has shown to be significantly effective in terms of anti-cancer management. Its superiority arises from its capacity to target many pathways, and reducing drug resistance to a minimum. Pathway dysregulation in cancer cells, as well as the alteration of homeostatic settings, all contribute to the unregulated proliferation. For example, mutations in tumor suppressor genes such as p53, which normally activates cell cycle arrest when DNA is damaged, resulting in the accumulation of damaged DNA and the inhibition of cell cycle arrest, contribute to an increase in the rate of cell proliferation. Additionally, in cancer cells, upregulated autocrine growth factor production or an upregulated autocrine loop can contribute to tumor cell growth [27]. In colon cancer cells, a similar effect can be seen [28]. When it comes to autocrine growth factors, if VEGF is upregulated it can lead to metastasis, which can make the prognosis for survival worse [22][29]. Therefore, targeting several pathways with a multiple-agent combination can enhance the treatment while lowering the risk of cancer cells becoming more aggressive and incurable. In addition, the doses of each drug/compound in combination therapy can be lowered, resulting in fewer adverse effects compared with monotherapy [30]. Another benefit of combination therapy is that different drugs can target the heterogeneous character of tumors, boosting the chances of killing cancer cells, including the cancer stem cell population, which has been linked to drug resistance and cancer recurrence following remission [31][32][33].

7. Conclusions
In conclusion, diosmetin in combination with 5-FU has a synergistic effect in HCT116 colon cancer cells and an additive effect in HT29 cells. Combination therapy enhances the efficacy of 5-FU and reduces its unfavorable adverse effects, which was demonstrated by high DRI and a high synergy score in HCT116 cells. The combination of 5-FU and diosmetin activates apoptosis mainly via the intrinsic pathway and arrests HCT116 cells at the G2/M phase. Further studies are required to assess the underlying mechanism of action of the combination, and to confirm the anti-tumorigenic activities of this combination in an appropriate animal model.

References
Keywords

synergistic effect; combination index; diosmetin; 5-fluorouracil; colorectal cancer; dose reduction index