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Cytotoxic and Apoptotic Effects of Carmofur and Vitamin C Combination on HCT116 Colon Cancer Cells

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ABSTRACT

Objective: In recent years, especially combined treatment options that will reduce the side effects of drugs have attracted attention. Carmofur is a new potent agent being investigated in the treatment of cancer. This study aimed to investigate the cytotoxic and apoptotic effects of carmofur+vitamin C combination in colon cancer cells.

Materials and Methods: Carmofur in the range of 7.8-250 µM, vitamin C in 7.8 µM-2mM and carmofur+vitamin C in different concentrations were evaluated on HCT116 cells by MTT test. In addition, the cell death pathway was determined by an apoptosis-necrosis assay.

Results: The IC_{50} value of carmofur in HCT116 cells was 8 µM, vitamin C was not effective at low doses, and the IC_{50} value was 2.2 mM. When carmofur+vitamin C were applied to HCT116 cells, 4 µM carmofur and 125 µM vitamin C together inhibited half of the cells (IC_{50}), while 4 µM carmofur and 2 mM vitamin C inhibited half of the cells similarly.

Conclusion: Vitamin C doubled the anticancer effect of carmofur. As a result, it was observed that vitamin C supplementation significantly increased cell death in anticancer drug administration. In addition, it was determined that the addition of vitamin C significantly increased the apoptotic activity.

Keywords: Carmofur, vitamin C, combined therapy, colon cancer, HCT116

INTRODUCTION

Cancer is the second cause of death in the world and an important health problem. Colon cancer, which is one of the most common malignant diseases in humans, is the third most common cause of cancer mediated mortality in the most developed countries and worldwide (1). Despite the numerous approaches to prevent and treat cancer, for instance, chemotherapy, surgery and radiotherapy, and some advances in cancer treatment, there is still a necessity to improve effective, leading-edge cancer treatments (2, 3). However, the widespread side effects and reactions of the drugs limit the effectiveness of current cancer treatments (4). Therefore, it is necessary to find new approaches and find novel and effective drugs to reduce side effects in cancer treatment (5). In recent years, one of the drugs researched in cancer treatment is carmofur, which is a ceramidase inhibitor and a new potent candidate in cancer therapy. Carmofur, a masked form of 5-Fluorouracil (FU), is a pyrimidine analog used as an antineoplastic agent (6). Unlike 5-FU, carmofur is destroyed by the extrahepatic route as well as the hepatic route and its level in the blood may increase to higher rates (7). Carmofur has also been used as adjuvant chemotherapy for breast and colorectal cancer in some countries (7, 8). In addition, it is a very strong acid ceramidase (AC) inhibitor and causes ceramide accumulation in cancer cells. Ceramide acts as a messenger that activates apoptosis and cell differentiation (9, 10). In addition, some

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researchers reported that carmofur was more effective in targeting 5-FU resistant cells (11). However, the exact mechanism of carmofur on cancers is not known.

Vitamin C is an essential nutrient that our body needs for normal function and it has anticancer and antioxidant activities. Although vitamin C cannot be synthesized by the body, it is plentiful in many natural sources and can be taken as a dietary supplement. Moreover, it scavenges reactive oxygen species (ROS) and induces cytotoxicity against tumor cells, prevents glucose metabolism, acts as an epigenetic regulator, and plays an important role in preventing cancer development by many mechanisms (4). In *in vitro* studies, it has been observed that high concentrations of vitamin C inhibit cell migration and angiogenesis (12). Accordingly, vitamin C can inhibit the metastasis and proliferation of tumor cells. In addition, vitamin C is not significantly toxic to normal cells (13) and is low-cost and readily available, so it may be an excellent candidate to improve an effective anticancer agent.

Numerous approaches and materials are being researched for dealing with cancer. In this context, carmofur, an anticancer drug whose efficacy is not fully known, has recently emerged as a potential candidate. In addition, in order to reduce the side effects of cancer drugs and increase their therapeutic effect, the combination of natural compounds such as vitamin C is being investigated as a treatment option.

This study focuses on the importance of vitamin C in reducing the side effects while increasing the cytotoxic effect of anti-cancer drugs. The present study aimed to develop a new, more effective possible treatment strategy, besides reducing the side effects of carmofur in colon cancer, and examined the effect of the combination of vitamin C with carmofur on colon cancer cells. In the light of this information, in this study, the cytotoxic effect and death pathway of carmofur and vitamin C in HCT116 colon cancer cells, separately and combined, were investigated.

MATERIALS AND METHODS

HCT116 colon cancer cell lines obtained from ATCC were used in the present study. Dulbecco's-Modified-Eagle-Medium (DMEM; Sigma, D2902), $NaHCO_3$ (Sigma, S5761), penicillin-streptomycin (10,000 U/mL, 15140148), Fetal Bovine Serum (FBS, Sigma F4135) was used to prepare cell media. Cells were removed with trypsin (Sigma, T9935). MTT (Sigma, M2128) and Dimethyl sulfoxide (DMSO; Merck, 102952) were used for cytotoxicity determination. Cancer cells were treated with vitamin C (Sigma, A4403) and carmofur (Glentham, GP5925). Annexin V-FITC apoptosis Kit (Beckman Coulter, IM2375) was used for the determination of apoptosis.

Cell Culture Studies

HCT116 cells were cultured at 37°C in an atmosphere of 95% O_2, 5% CO_2, DMEM containing 100.000 U/L penicillin, 100.000 g/L streptomycin, 10% FBS was used as the medium. The medium of the cells was refreshed at least twice a week. Cells were passaged when they were 70-90% confluent.

Evaluation of Cell Viability by MTT Test

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) is a quantitative, colorimetric method which is used to quantitate cytotoxic activity based-on *in vitro* metabolism viability (14). In this study, stock solutions of the drugs were prepared for carmofur and vitamin C in dimethylsulfoxide (DMSO) (with DMSO ratio below 1%). Carmofur and vitamin C were then diluted to different concentrations with DMEM and incubated with HCT116 colon cancer cells for 72 hours in a 5% CO_2 incubator. 96-well culture plates were used and 90 µl of the cells 10^5 per milliliter were taken into wells. Then, carmofur was added in the range of 7.8-250 µM, vitamin C in the range of 7.8 µM-2 mM, and carmofur+vitamin C combined in different concentrations and added 10 µl separately to wells on the plate and incubated for 72 hours. After incubation, MTT (5 mg/ml, PBS) was added and incubated for 3 hours, and the absorbance was measured at 570 nm in an ELISA plate reader. According to dose/response curves, half-maximal inhibitory concentrations (IC_{50}) of the compounds were determined. Each test was repeated at least 3 times.

Determination of Apoptosis/Necrosis

In HCT116 cells, the rates of viable, apoptotic/necrotic cells were evaluated via the annexin V-FITC/propidium iodide. HCT116 cells were cultured in a 6-well plate as 3×10^5 cells per well with IC_{50} doses of the compounds. Evaluation of apoptosis and necrosis was performed at IC_{50} doses and for 72 hours to be compatible with MTT tests: Carmofur 8 µM, vitamin C 2.2 mM, carmofur (4 µM) + vitamin C (250 µM), carmofur (4 µM) + vitamin C (2 mM) concentrations were prepared for combination treatments. These concentrations were added to each well and incubated for 72 h. The cells were centrifuged for 10 minutes. The cell pellet was washed with 1 mL of PBS and centrifuged again. This procedure was repeated 3 times. Then, 100 µL of the cell pellet was stained by 5 µL annexin V-FITC and 2.5 µL propidium iodide. After 15 minutes, cells were measured on a Beckman Coulter flow cytometer, showing viable, early apoptosis (Annexin positive/PI negative), and late apoptosis (both Annexin/PI-positive) cells. The results were evaluated with the Kaluza analysis program.

Statistical Analysis

Data were first evaluated for normality via Shapiro-Wilk test. Samples were compared with one-way analysis of the variance (ANOVA) test, and Tukey, as a post-hoc-test, was utilized. $p<0.05$ was considered as statistical significance level and analyses were evaluated with GraphPad software.

RESULTS

Cytotoxic Effects of Carmofur and Vitamin C on HCT116 Cells

HCT116 colon cancer cells were incubated with carmofur alone, vitamin C alone, and combined concentrations of the two compounds. When carmofur was applied to colon cancer cells at 7.8, 15.6, 31.5, 62, 125 and 250 µM concentrations, the cell viability was 51%, 48%, 42%, 32%, 35%, and 26%, respec-

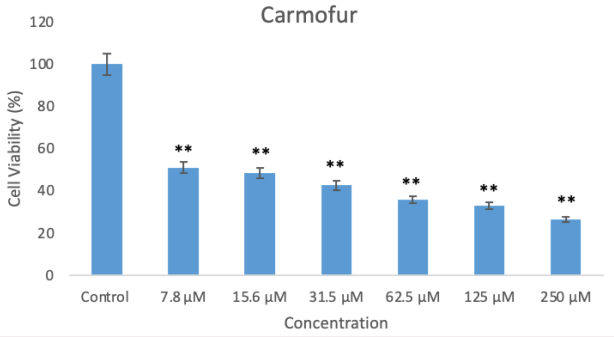

Figure 1. Cell viability effect of different concentrations of carmofur on HCT116 colon cancer cells. Cell viability tests were evaluated by MTT test after 72 hours of incubation. Experiments were repeated at least 3 times.

Statistical significance is shown with **p<0.01 compared to the untreated control group.

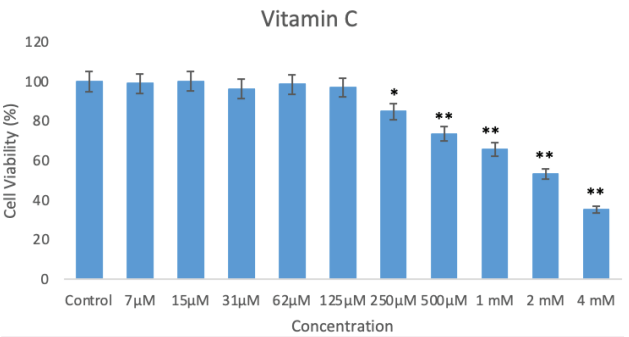

Figure 2. Cell viability effect of different concentrations of vitamin C on HCT116 colon cancer cell lines. Cell viability tests were evaluated by MTT test after 72 hours of incubation.

Statistical significance is shown by *p<0.05 and **p<0.01 compared to the untreated control group.

tively. When vitamin C is applied at 7.8, 15.6, 31.5, 62, 125 and 250, 500 µM, 1 mM, 2 mM and 4 mM concentrations, the viability of colon cancer cells was determined as 98%, 99%, 96%, 98%, 96%, 94%, 84%, 73%, 64%, 53% and 35% respectively (Figure 1, Figure 2).

It was observed that low-doses of vitamin C alone were not effective in cancer cells. The IC_{50} value of carmofur alone on colon cancer cells was determined at 8 µM, and the IC_{50} value of vitamin C alone was 2.2 mM. When carmofur and vitamin C were applied as a combination, 4 µM carmofur+125 µM vitamin C inhibited half of the cells (IC_{50}), while 4 µM carmofur+2 mM vitamin C had a similar effect (Figure 1, Figure 2, Figure 3, Figure 4).

Combined Cytotoxic Effects of Carmofur and Vitamin C on HCT116 cells

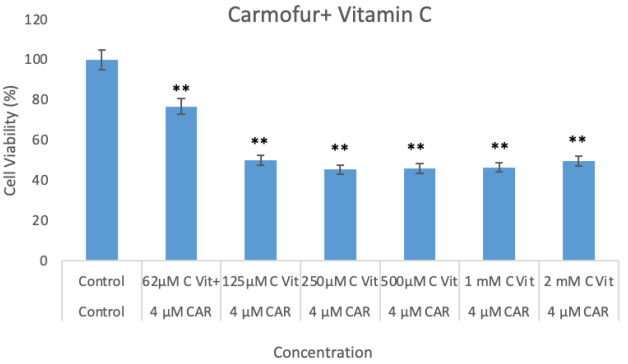

Figure 3. Cell viability effect of 4 µM carmofur concentration and different concentrations of vitamin C combination on HCT116 cells. Cell viability tests were evaluated by MTT test after 72 hours of incubation.

Statistical significance is shown with **p<0.01 compared to the drug-free control group. CAR: Carmofur.

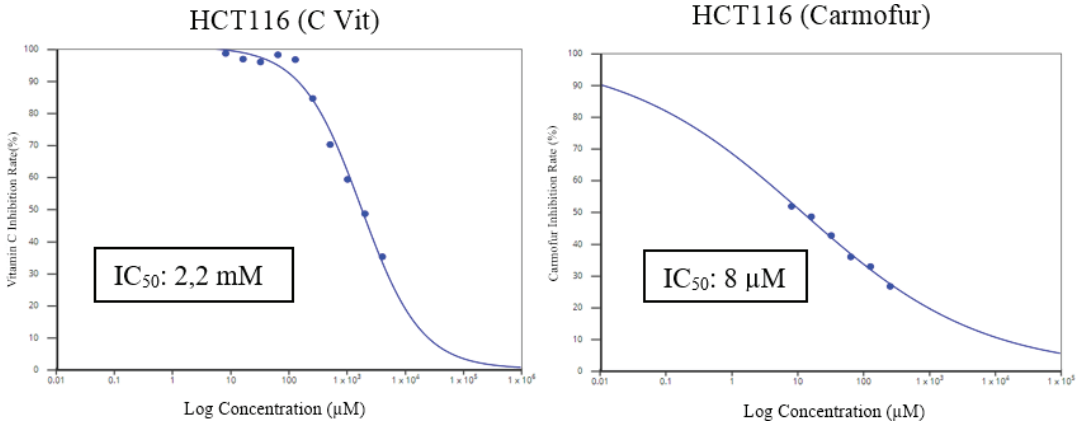

Figure 4. IC_{50} values of vitamin C and carmofur on HCT116 cells. Calculations were performed with "AAT bioquest IC_{50} calculator" program.

IC_{50}: Concentration that inhibits 50% of the cells.

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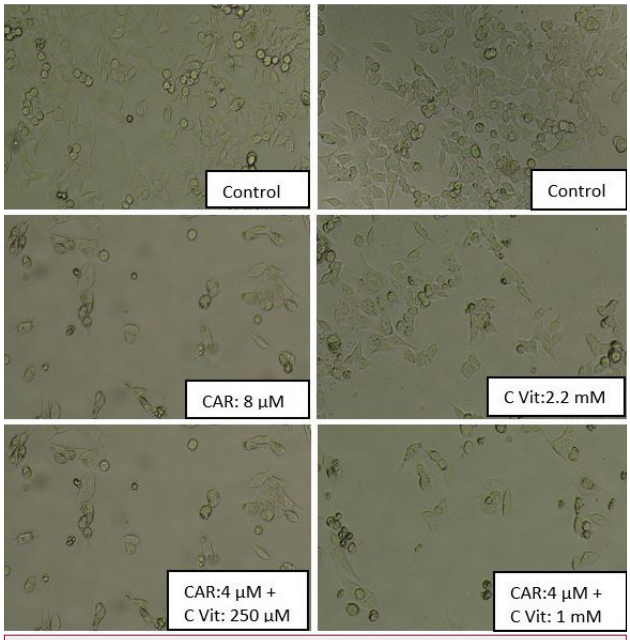

Figure 5. Morphological changes on HCT116 cells when carmofur and vitamin C were administered alone or together (Light microscope: 200X) (CAR: Carmofur).

Figure 5 shows the morphological changes in the untreated control group and treated HCT116 cells. It is observed that the cells of the untreated control group are fusiform, connected to each other, and adhered to the surface. There was a remarkable decrease in the number of cells in the groups where carmofur and vitamin C were administered together or alone. Shrinkage and rounding are observed in some cells, and the decrease in cell number and intercellular connections is noteworthy. In figure 5, images of the treated groups with approximate IC_{50} values are given. According to the findings obtained in this study, morphological changes are compatible with cytotoxicity and apoptosis tests.

Evaluation of Apoptotic-Necrotic Effects by Flow Cytometry Analysis on HCT116 Cells

Figure 6 shows the apoptotic and necrotic effects of carmofur and vitamin C alone or combined in HCT116 cancer cells. To see cell death in the apoptosis-necrosis assay, concentrations that inhibited about half of the cells (IC_{50}) were studied. Apoptosis-necrosis findings were consistent with cytotoxicity findings (Figure 6).

In addition, when we evaluated the apoptotic effect of carmofur alone at the IC_{50} concentration, 51% of the cells were alive, while 11% were under early apoptosis, 23% late apoptosis, and

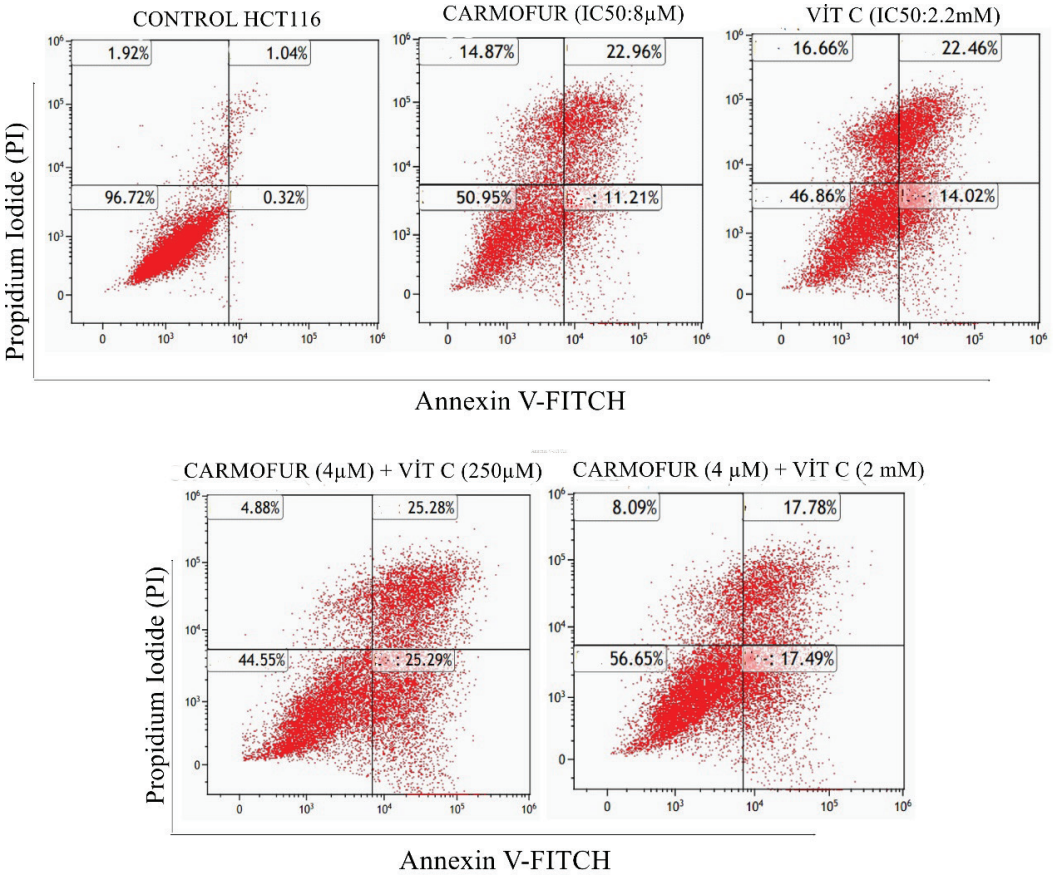

Figure 6. Flow cytometry analyses of HCT116 cells after incubations with C armofur and vitamin C alone and combined.

Table 1. Apoptotic necrotic rates (%) of carmofur alone, vitamin C alone, and carmofur+vitamin C combination on HCT116 colon cancer cells.

	Live (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis/Dead (%)
Control	96.72	0.32	1.04	1.92
Carmofur	50.95	11.21	22.96	14.87
Vitamin C	46.86	14.02	22.56	16.66
Carmofur (4 µM) + vitamin C (250 µM)	44.55	25.29	25.28	4.88
Carmofur (4 µM) + vitamin C (2 mM)	56.65	17.49	17.78	8.09

14% necrosis. When we evaluated the apoptotic effect of vitamin C alone at the IC_{50} concentration, 47% of the cells were alive, while 14% had early apoptosis, 22% had late apoptosis, and 16% had necrosis. Moreover, as a result of the combination of carmofur with low dose vitamin C (250 µM), it caused 45% cell viability, 25% early apoptosis, 25% late apoptosis, 4% necrosis, while vitamin C is used in high doses (2.2 mM) combined, 56% viable cells, 17% early apoptosis, 17% late apoptosis, 8% necrosis rate were observed (Figure 6, Table 1). According to these findings, when carmofur was used in combination with vitamin C, it was determined that apoptosis on cancer cells increased significantly.

DISCUSSION

In this study, the cytotoxic effects of carmofur and vitamin C at different concentrations on HCT116 colon cancer cells were evaluated. Carmofur, an acid ceramidase inhibitor (9), was quite effective in these cells, and the IC_{50} value was found to be 8 µM. For vitamin C, µM and mM concentrations were studied. Vitamin C alone did not show significant cytotoxicity in colon cancer cells at doses of 250 µM and below. The IC_{50} value obtained by the application of vitamin C alone, which is the concentration that inhibits 50% of the cells, was determined to be 2.2 mM (Figure 1, Figure 2). In addition, when 4 µM, which is half of the IC_{50} concentration of carmofur, and different concentrations of Vitamin C (in the range of 62 µM- 2 mM) are combined, it is observed that especially the concentrations of 125 µM vitamin C and above significantly increase cytotoxicity **(**Figure 3**).** Half of the HCT116 cells were inhibited (IC_{50}) when 4 µM carmofur (half the IC_{50} value) was co-administered with 125 µM vitamin C. In other words, with the support of vitamin C, the concentration of the chemotherapy drug decreased by half and showed the same effect. Vitamin C and carmofur showed a synergistic effect. Interestingly, similar cytotoxicity was detected when the vitamin C concentration was increased up to 2mM in combination treatment with carmofur.

Most interestingly, while vitamin C alone shows toxicity at high doses such as 2 mM, when used in combination with carmofur anticancer drug, it reaches the highest cytotoxicity at 125 µM concentrations, and there is no significant difference in combination between high doses such as 1mM and 2mM. While 8 µM carmofur had the IC_{50} value, 4 µM carmofur + 125 µM vitamin C shows the same effect. Similar to this study, Ghavami et al. reported that when they used vitamin C and cisplatin in combination, the addition of vitamin C increased cell death in gastric cancer cells (15). In another study, Lee et al. evaluated the combined effect of different anticancer drugs such as tamoxifen, fulvestrant, trastuzumab, and vitamin C on MCF-7, and MDA-MB-231 breast cancer cells. It has been reported that the combined use of vitamin C and anticancer drugs provides a therapeutic advantage in breast cancer cells (16).

The development of effective pro-apoptotic agents with drug discovery studies and understanding the mechanism are of great importance for cancer treatment (17). In this study, it is clearly seen that combined treatment with vitamin C triggers apoptotic activity. When we evaluate the apoptotic effect, when carmofur and vitamin C are applied alone, it is seen that some necrosis is triggered along with apoptosis **(**Figure 6, Table 1**).** When low dose (250 µM) vitamin C is applied combined with carmofur, it is seen that apoptotic effect is increased significantly (50%) and necrosis is decreased (4%). In addition to stimulation of apoptosis, vitamin C alone at a high dose (2.2 mM) also caused significant necrosis. However, when carmofur + vitamin C was combined, especially with low-dose vitamin C (250 µM), it is noteworthy that apoptosis increased significantly. According to these results, the addition of vitamin C in anticancer drug use induces apoptosis in cell death. Although there are some studies investigating the anticancer effect of carmofur alone, these are very limited, moreover, there is no study based on the use of carmofur together with vitamin C in the literature. In a study by Çömlekçi et al., the IC_{50} value of carmofur was 16 µM in A549 lung cancer cells (18), while it was reported in the range of 4.6-50 µM in pediatric brain tumors in another study (19). The cytotoxic activity found in this study is also consistent with other studies.

The high level of acid ceramidase in cancer cells increases resistance to treatment and complicates treatment (10). Carmofur is a very strong acid ceramidase inhibitor and can reduce

drug resistance, which is the biggest problem in treatment, by inhibition of acid ceramidase, especially in treatment-resistant cancers such as colon cancer (20). For this reason, in this study, it was shown that carmofur has a very strong anticancer effect on HCT116 cells, which are highly resistant to treatment, and this effect can be further enhanced with vitamin C. When we compared the effect of other anticancer drugs in many studies conducted to date, we showed that carmofur can be a powerful anticancer drug for colon cancer and this effect is multiplied by vitamin C.

Vitamin C and its derivatives are compounds that need further investigation with their potential to aid treatment. Vitamin C and its derivatives can offer important therapeutic options in cancer treatment with their advantages such as easy availability, low toxicity and low cost. However, further studies are needed on the dose and clinical benefits of vitamin C in cancer treatment.

CONCLUSION

This study demonstrates that co-administration of vitamin C and carmofur could be developed in the future as an innovative, effective therapeutic strategy for colon cancer patients. The combination of carmofur with vitamin C significantly increased cytotoxicity and apoptotic activity in colon cancer cells.

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Ethics Committee Approval: Ethics committee approval is not required because of no material or experimental animal that would require permission.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The author has no conflict of interest to declare.

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REFERENCES

1. Razali FN, Sinniah SK, Hussin H, Abidin NZ, Shuib AS. Tumor suppression effect of Solanum nigrum polysaccharide fraction on Breast cancer via immunomodulation. Int J Biol Macromol 2016; 92: 185-93. [CrossRef]
2. Sharma PC, Bansal KK, Sharma A, Sharma D, Deep A. Thiazole-containing compounds as therapeutic targets for cancer therapy. Eur J Med Chem 2020; 188: 112016. [CrossRef]
3. Sharma PC, Sharma D, Sharma A, Bhagat M, Ola M, Thakur VK, et al. Recent advances in microbial toxin-related strategies to combat cancer. In Seminars in Cancer Biology. Academic Press 2021. [CrossRef]
4. Reang J, Sharma PC, Thakur VK, Majeed J. Understanding the therapeutic potential of ascorbic acid in the battle to overcome cancer. Biomolecules 2021; 11(8): 1130. [CrossRef]
5. Miao Y, Xiao B, Jiang Z, Guo Y, Mao F, Zhao J. Growth inhibition and cell-cycle arrest of human gastric cancer cells by Lycium barbarum polysaccharide. Med Oncol 2010; 27(3):785-90. [CrossRef]
6. Zhang P, Liu S. Solvent Effects on the UV Absorption Spectrum of Carmofur. Detection 2016; 4: 25-31. [CrossRef]
7. Ken M, Masae K. Postoperative adjuvant use of carmofur for early breast cancer. Osaka City Med J 2003; 49(2):77-83.
8. Nakamura T, Ohno M, Tabuchi Y, Kamigaki T, Fujii H, Yamagishi H, et al. Optimal duration of oral adjuvant chemotherapy with Carmofur in the colorectal cancer patients: the Kansai Carmofur Study Group trial III. Int J Oncol 2001; 19(2): 29198. [CrossRef]
9. Realini N, Solorzano C, Pagliuca C, Pizzirani D, Armirotti A, Luciani R, et al. Discovery of highly potent acid ceramidase inhibitors with in vitro tumor chemosensitizing activity. Sci Rep 2013; 3: 1035. [CrossRef]
10. Clifford RE, Govindarajah N, Bowden D, Sutton P, Glenn M, Darvish-Damavandi M, et al. Targeting Acid Ceramidase to Improve the Radiosensitivity of Rectal Cancer. Cells 2020; 9(12): 2693. [CrossRef]
11. Maehara Y, Kusumoto H, Anal H, Kusumoto T, Hiramoto Y, Sugimachi K. 1-Hexylcarbamoyl-5-fluorouracil is more cytostatic than 5-fluorouracil against human tumors in vitro. Eur J Cancer Clin Oncol 1987; 23(10): 1511-5. [CrossRef]
12. Mikirova NA, Ichim TE, Riordan NH. Anti-angiogenic effect of high doses of ascorbic acid. J Transl Med 2008; 6(1): 1-10. [CrossRef]
13. Lee WJ. The prospects of vitamin C in cancer therapy. Immune Netw 2009; 9(5): 147-52. [CrossRef]
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983; 65(1-2): 55-63. [CrossRef]
15. Ghavami G, Sardari S. Synergistic Effect of Vitamin C with Cisplatin for Inhibiting Proliferation of Gastric Cancer Cells. Iran Biomed J. 2020; 24(2): 119-27. [CrossRef]
16. Lee SJ, Jeong JH, Lee IH, Lee J, Jung JH, Park HY, et al. Effect of high-dose vitamin C combined with anti-cancer treatment on breast cancer cells. Anticancer Res 2019; 39(2): 751-8. [CrossRef]
17. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol 2020; 17(7): 395-417. [CrossRef]
18. Çömlekçi E, Kutlu HM, Sezer CV. Toward stimulating apoptosis in human lung adenocarcinoma cells by novel nano-carmofur compound treatment. Anti-cancer drugs 2021; 32(6): 657-63. [CrossRef]
19. Doan NB, Nguyen HS, Montoure A, Al-Gizawiy MM, Mueller WM, Kurpad S, et al. Acid ceramidase is a novel drug target for pediatric brain tumors. Oncotarget 2017; 8(15): 24753. [CrossRef]
20. Dementiev A, Joachimiak A, Nguyen H, Gorelik A, Illes K, Shabani S, et al. Molecular mechanism of inhibition of acid ceramidase by carmofur. J Med Chem 2018; 62(2): 987-92. [CrossRef]