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Growth-Inhibitory Effects of Traditional Chemotherapeutic Combinations (FOLFOX, FOLFIRI) on Colon Cancer Cells: An in vitro Study

Geleneksel Kemoterapötik Kombinasyonların (FOLFOX, FOLFIRI) Kolon Kanseri Hücreleri Üzerindeki Büyüme-Engelleyici Etkileri: Bir in vitro Çalışma

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ABSTRACT

Objective: To compare the *in vitro* growth-inhibitory effects of the commonly used chemotherapeutic combinations folinic acid, 5-fluorouracil, and oxaliplatin (FOLFOX) and folinic acid, 5-fluorouracil, and irinotecan (FOLFIRI) on colon cancer cells (HT29 and CaCo-2 cells).

Materials and Methods: The viability of HT29 and CaCo-2 cells treated with different concentrations of the FOLFOX and FOLFIRI combinations was evaluated using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay.

Results: FOLFOX and FOLFIRI combinations exhibited varying effects on the colon cancer cell lines, with HT29 cells showing sensitivity, while CaCo-2 cells demonstrated resistance to these treatments.

Conclusions: The results of this preliminary study will contribute to the development of effective and targeted clinical treatment strategies for colon cancer.

Keywords: Colon cancer, drug combination, FOLFOX, FOLFIRI, growth-inhibitory effect

ÖZ

Amaç: Yaygın olarak kullanılan kemoterapötik kombinasyonlar olan folinik asit, 5-florourasil ve oksaliplatin (FOLFOX) ile folinik asit, 5-florourasil ve irinotekanın (FOLFIRI) kolon kanseri hücreleri (HT29 ve CaCo-2 hücreleri) üzerindeki in vitro büyümeyi inhibe edici etkilerini karşılaştırmak.

Materyal ve Metot: FOLFOX ve FOLFIRI kombinasyonlarının farklı konsantrasyonlarıyla tedavi edilen HT29 ve CaCo-2 hücrelerinin canlılığı MTT (3-(4,5-dimetiltiazolil-2)-2,5-difeniltetrazolium bromür) testi kullanılarak değerlendirildi.

Bulgular: FOLFOX ve FOLFIRI kombinasyonları kolon kanseri hücre hatları üzerinde farklı etkiler gösterdi, HT29 hücreleri duyarlılık gösterirken, CaCo-2 hücreleri bu tedavilere direnç gösterdi.

Sonuç: Bu ön çalışmanın sonuçları, kolon kanseri için etkili ve hedefli klinik tedavi stratejilerinin geliştirilmesine katkıda bulunacaktır.

Anahtar Kelimeler: Antiproliferatif etki, FOLFIRI, FOLFOX, ilaç kombinasyonu, kolon kanseri

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INTRODUCTION

Cancer, which is one of the leading causes of death worldwide, is characterized by aberrant cell growth and spread. Colorectal cancer is the most common malignant tumor of the gastrointestinal tract and accounts for approximately 10%–15% of all cancer types.[1] The etiology of colorectal cancer involves the accumulation of tumor suppressor gene mutations in epithelial cells of the large intestine (also called the colon) or the rectum (the terminal part between the anus and intestine).[2] However, traditional therapeutic methods, such as chemotherapy, radiotherapy, and surgical resection, have some limitations, including low treatment compliance, low accuracy, high toxicity, and drug resistance.[3] The application of the combination of traditional chemotherapeutic drugs is a promising strategy to increase treatment efficacy and mitigate side effects.[4] For example, combinatorial therapies, such as the folinic acid (FA), 5-fluorouracil (5-FU), and oxaliplatin (OX) (FOLFOX) combination and the FA, 5-FU, and irinotecan (IRI) (FOLFIRI) combination are usually used for cancer treatment. These drug combinations target cancer cells through interaction with cancer cell components and mitigate drug resistance development.[5]

FOLFOX, which is a widely used therapeutic combination for colon cancer, comprises FA, 5-FU, and OX. OX, a platinum-based compound, inhibits DNA replication and transcription by cross-linking with DNA and consequently induces cellular apoptosis.[6] Meanwhile, 5-FU, a pyrimidine analog, inhibits the proliferation of cancer cells by interfering with nucleic acid metabolism, which has critical roles in various stages of the cell cycle.[7] Folinic acid potentiates the efficacy of 5-FU.[8,9] The second most commonly preferred chemotherapeutic combination for colon cancer is FOLFIRI, which comprises FA, 5-FU, and IRI.[10] IRI inhibits topoisomerase I, which unwinds the DNA helix during DNA replication, resulting in the induction of DNA breaks and cell death.[11] Thus, FOLFOX and FOLFIRI combinations are effective therapeutics for colon cancer.[12]

This study was carried out to evaluate the effectiveness of various chemotherapy drugs used in clinical settings through a polychemotherapeutic approach. This preliminary research lays the groundwork for advanced preclinical studies and potential clinical applications at the cellular and molecular levels. The main objective is to examine the cellular effects of each drug combination (FOLFOX and FOLFIRI) to optimize treatment strategies.

MATERIALS AND METHODS

Ethics Committee Approval: This research utilized commercially available cell lines. As such, no ethics committee approval was necessary for this study. The research adhered to international declarations and guidelines.

Preparation of Chemicals: IRI (I-4122, LC Laboratories, Woburn, MA, USA) and 5-FU (F6627, Sigma Aldrich, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich), aliquoted, and stored at −80 °C. To mitigate the cytotoxic effect of DMSO, the concentration of DMSO in the culture medium was maintained at <0.1%. FA (PHR1541, Sigma Aldrich) and OX (O-7111, LC Laboratories, Woburn, MA, USA) were dissolved in distilled water and stored at −80 °C. As FA is light-sensitive and labile, it was freshly prepared before the experiment.

Cell Culture Studies: The colon cancer cell lines (HT29 and CaCo-2 cells) were obtained from the stocks of XXX, Faculty of Pharmacy, Animal Cell and Tissue Culture Laboratory. Cells were cultured in culture flasks with a medium (RPMI 1640 for HT29, EMEM for CaCo-2) containing 10% inactivated fetal bovine serum (F0926, Sigma-Aldrich, USA), 1% L-glutamine (25030024, Gibco, USA), and 0.1% gentamicin (G1337, Sigma Aldrich) at 37 °C and 5% CO_2 in an incubator. Cultured HT29 and CaCo-2 cells were trypsinized using a trypsin-EDTA solution (T4049, Sigma Aldrich). The cells were transferred to 96-well culture plates (initial cell concentration = 5×10^4 cells/mL) in four replicates and cultured for 24 h in a humidified incubator at 37 °C and 5% CO_2.

Cell Viability Analysis: HT29 and CaCo-2 colon cancer cell lines were treated with 5-FU, FA, OX, and IRI, as well as their combinations (FOLFOX and FOLFIRI). An initial concentration of 100 µM was used, followed by serial dilution to create 7 different concentrations (1.56, 3.125, 6.25, 12.5, 25, 50, 100 µM). Cells were incubated with the chemotherapeutic agents for 72 hours, and viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, USA) assay at 24-hour intervals. After the incubation period, absorbance was measured at 570 nm using an ELISA reader. The data of the treatment groups were compared with those of the control group to obtain the half-maximal inhibitory concentration (IC_{50}) values using GraphPad Prism® version 9.0.0 software. The cell viability percentage graphs were generated by comparing the growth-inhibitory effects of chemotherapy drugs and their combinations on HT29 and CaCo-2 cell lines with those of the negative control. The cell viability percentage was calculated as follows: (% viability = ¼ A samples / A negative control x 100).[13]

Statistical Analysis: The data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism® version

9.0.0. Means were compared using a two-way analysis of variance, followed by multiple comparison post-hoc tests.

RESULTS

The responses of the groups treated with chemotherapeutic drugs and their combinations were compared with those of the negative control group. Percentage viability graphs were generated for each group. All values are presented as mean ± SD. The values of the treatment groups were normalized to those of the negative control group.

As seen in Table 1, it was determined that IC_{50} values decreased with time in both HT29 and CaCo-2 cell lines. This reveals that the drugs we use show increasing effectiveness over time and can inhibit cell viability at lower doses. There is a significant decrease, especially when the IC_{50} values at the 72nd hour are compared to the 24th hour. Additionally, the table shows that the IC_{50} value of FA cannot be calculated. FA is an agent used together with 5-FU in chemotherapy and increases the effectiveness of 5 -FU, and it does not have a cytotoxic effect on its own.

Table 1. Half-maximal inhibitory concentration (IC_{50}) values.

	CaCo-2			HT-29		
Drug (µM)	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
5-Fluoroucil (5-FU)	129.2 ± 5.65	71.49 ± 3.54	45.43 ± 2.43	120.4 ± 7.81	50.25 ± 3.12	12.35 ± 1.01
Folinic Acid (FA)	*	*	*	*	*	*
5-FU+FA (FF)	76.37 ± 1.42	52.46 ± 1.99	29.58 ± 0.09	109.1 ± 2.04	27.59 ±2.01	5.767 ± 0.84
Oxaliplatin (OX)	141.2 ± 3.78	11.46 ± 0.87	3.846 ± 1.05	46.02 ± 1.80	4.886 ± 1.044	2.058 ± 0.02
Irinotecan (IRI)	131.1 ± 7.60	53.81 ± 2.56	12.6 ± 1.22	63.71 ± 1.93	14.32 ± 2.06	5.974 ± 1.04
FOLFIRI (FF+IRI)	111.9 ± 7.53	21.01 ± 1.03	6.192 ± 0.78	60.66 ± 0.29	10.59 ± 1.021	3.763 ± 0.42
FOLFOX (FF+OX)	106.2 ± 3.41	7.196 ± 1.17	2.434 ± 0.19	42.21 ± 2.61	3.386 ± 1.90	1.036 ± 0.03

*: The viability of cells in the treatment groups marked with is higher than in the negative control, so the inhibition value could not be calculated; h: hour.

In the first step of the study, dose-response curves were generated for each chemotherapeutic agent in the HT29 and CaCo-2 colon cancer cell lines, as seen in Figure 1(a). In order to evaluate the potential antiproliferative effects of the chemotherapeutic agents on cancer cells, HT29 and Caco-2 cells were exposed to various concentrations of the test compounds. An initial concentration of 100 µM was used for each drug, followed by serial dilutions.[14] After 72 hours of incubation, cell viability was then evaluated using the MTT assay, as described above. It was observed that the two cell lines responded differently to the treatments due to inherent biological variability. Specifically, FA alone did not significantly affect cell viability across the tested concentrations, as seen in the flat dose-response curve. In contrast, 5-FU, OX, and IRI reduced cell viability in a dose-dependent manner, with HT29 cells generally showing greater sensitivity than Caco-2 cells.

Figure 1(b) shows the effect of FA and 5-FU on cell viability at the 72nd hour IC_{50} concentration in HT29 and CaCo-2 cell lines. As seen in the figure, using 5-FU together with FA increased the chemotherapeutic effect of 5-FU. Statistical analysis confirmed that the decrease in cell viability with the FF combination was significantly greater than the reduction observed with 5-FU or FA alone. Therefore, in this study, FA and 5-FU were evaluated together as a monochemotherapy agent, FF.

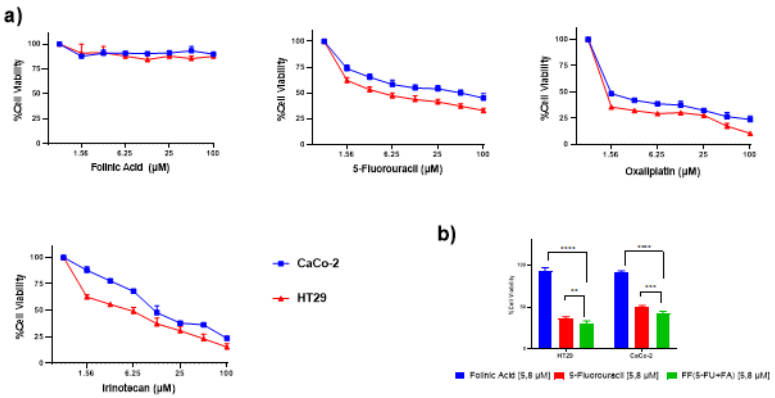

Figure 1. Optimization of Folinic Acid and 5-Fluorouracil Combination and Dose-Response Curves. a: Dose-response curves for folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin in CaCo-2 and HT29 cells after 72 hours of treatment; b: Effect of IC50 values of folinic acid and 5-fluorouracil on cell viability after 72 hours; Results were analyzed using a two-way ANOVA test; ****: $p \leq 0.0001$; ***: $p \leq 0.0005$; **: $p \leq 0.01$; *: $p \leq 0.05$ vs. FA.

Figure 2 and Figure 3 present the percentage viability graphs of HT29 and CaCo-2 cell lines, respectively, following treatment with FOLFOX and FOLFIRI combinations at various time points (24, 48, and 72 hours) and doses determined under laboratory conditions. The data indicate that combining chemotherapy drugs is significantly more effective than using them separately, particularly in HT29 cells, allowing for lower doses and reduced toxicity. While a significant decrease in cell viability was observed even during the 24-hour treatment period, it was determined that this decrease became more pronounced after 48 and 72 hours. This enhanced effect allows for the use of lower drug doses while maintaining therapeutic efficacy, which can help minimize the toxic side effects typically associated with higher doses. When Figure 3 was examined, it was seen that the effectiveness of both chemotherapy combinations was more limited in CaCo-2 cells. Despite prolonged treatment times, it was determined that the viability rates of CaCo-2 cells remained higher compared to HT29 cells. These findings reinforce the importance of considering both drug combination and cell line variability when designing chemotherapeutic regimens.[15]

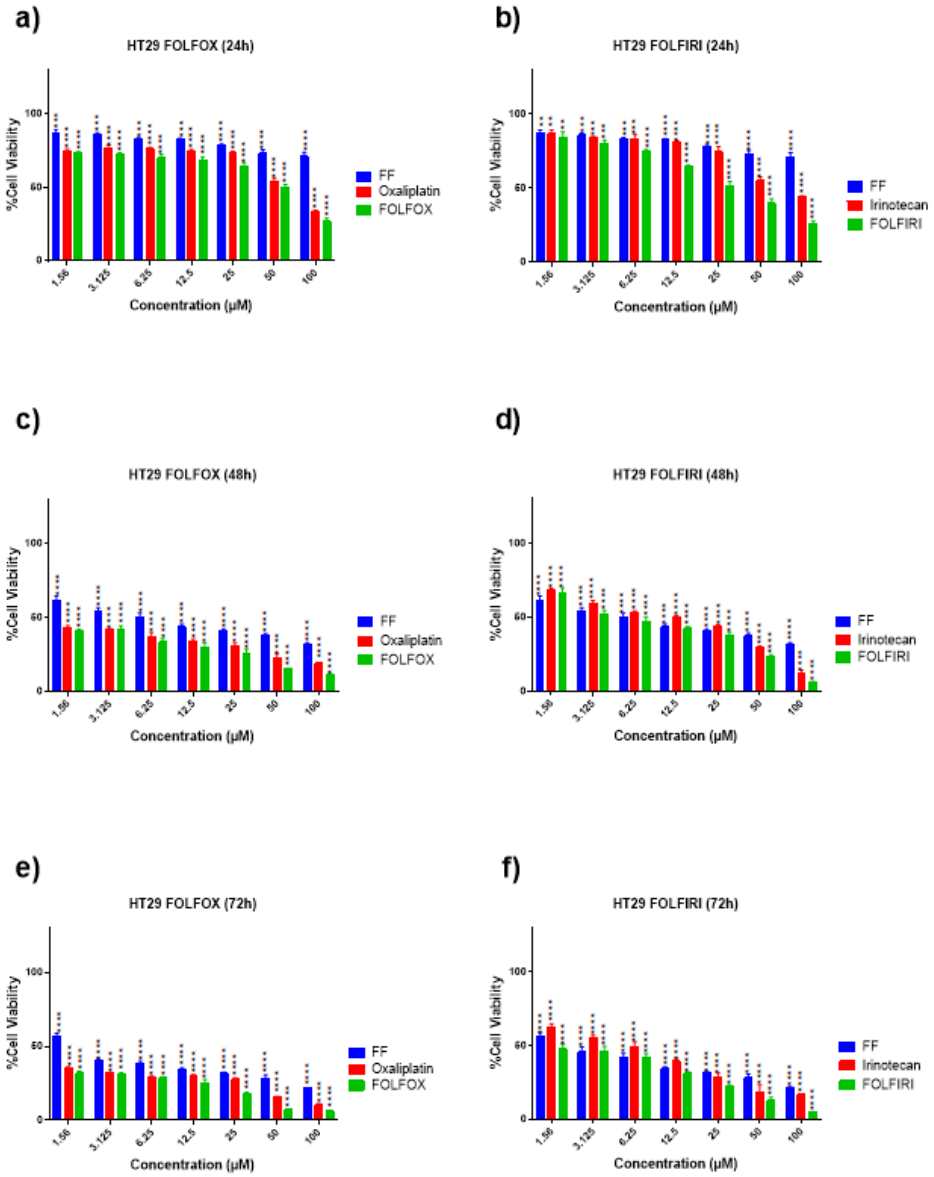

Figure 2. Percentage viability of HT29 cells after treatment with 5-fluorouracil + folinic acid (FF), oxaliplatin, irinotecan, (FF + oxaliplatin (FOLFOX)), and (FF + irinotecan (FOLFIRI)). a and b: 24 hours; c and d: 48 hours; e and f: 72 hours; Results were analyzed using a two-way ANOVA test; ****: $p \leq 0.0001$; ***: $p \leq 0.0005$; **: $p \leq 0.01$; *: $p \leq 0.05$ vs. Negative Control.

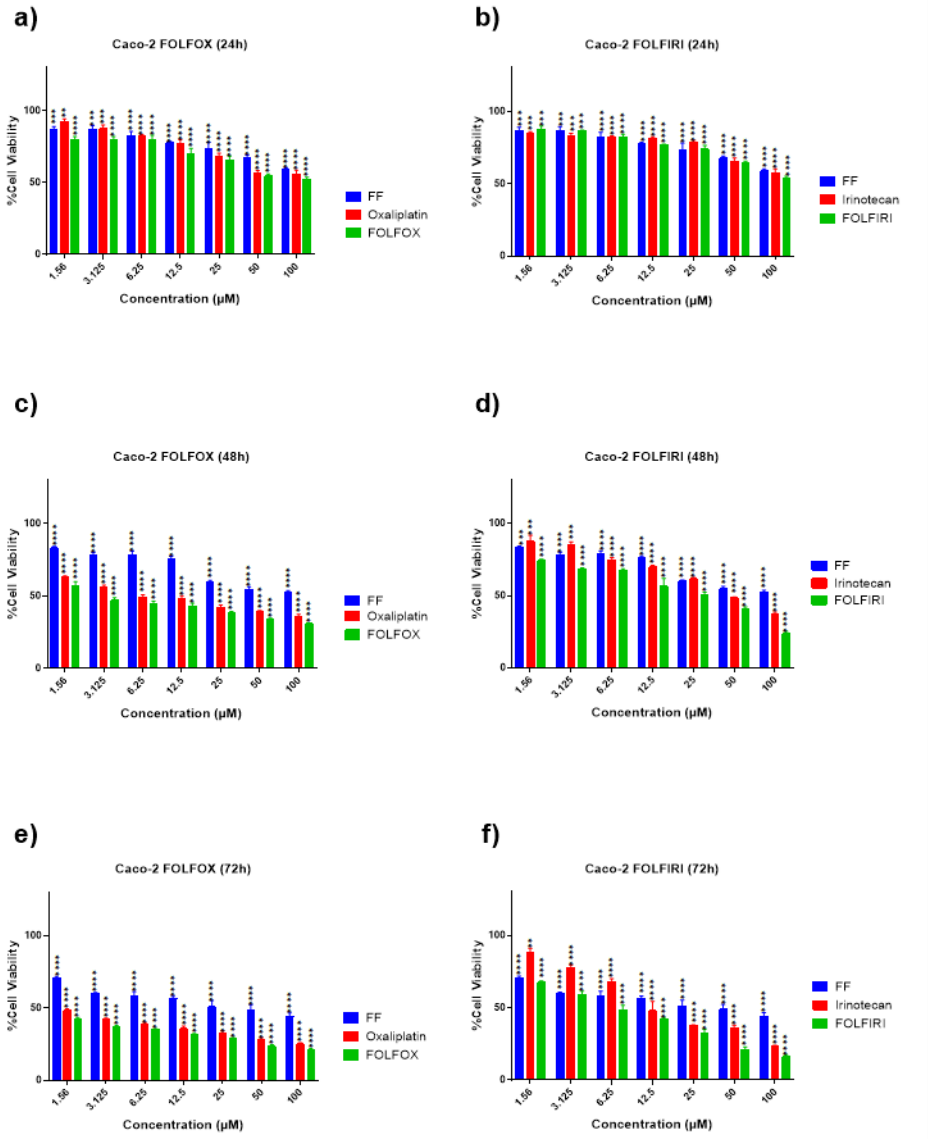

Figure 3. Percentage viability of CaCo-2 cancer cells treated with 5-fluorouracil (FF), oxaliplatin, irinotecan, (FF + oxaliplatin (FOLFOX)), and (FF + irinotecan (FOLFIRI)). a and b: 24 hours; c and d: 48 hours; e and f: 72 hours; Results were analyzed using a two-way ANOVA test; ****: $p \leq 0.0001$; ***: $p \leq 0.0005$; **: $p \leq 0.01$; *: $p \leq 0.05$ vs. Negative Control.

DISCUSSION AND CONCLUSION

The most common therapeutic modality for cancer is chemotherapy, which involves the usage of one or more cytotoxic drugs. As cancer cells divide faster than healthy cells, they are highly sensitive to cytotoxic compounds. However, rapidly multiplying healthy cells, such as bone marrow and hair follicle cells, are also sensitive to cytotoxic compounds. Thus, the common side effects of chemotherapeutic drugs include decreased red blood cell count and hair loss.

Chemotherapy is the mainstay treatment option for cancer despite side effects and the potential development of drug resistance, especially in advanced stages of cancer. Thus, efforts are ongoing to develop effective and tolerable anticancer drugs to mitigate drug resistance and side effects associated with chemotherapy.[16] Combinatorial therapy, which involves the application of a combination of chemotherapeutic drugs, is a promising treatment modality to suppress drug resistance and side effects, enhancing the therapeutic effect. This study aimed to provide evidence for developing new targeted treatments by comparing the effects of chemotherapeutic combinations on HT29 and CaCo-2 colon cancer cells. The IC_{50} values of drugs against HT29 cells were lower than those against CaCo-2 cells, indicating increased sensitivity of HT29 cells to chemo-

therapeutic drugs.[17] Consistent with previous findings, this study demonstrated that combination therapy was effective at low doses of drugs, which can prevent high-dose-induced toxicity.[18] Additionally, the drugs used in combination therapy prevent the division and proliferation of cancer cells through different mechanisms.[19] FOLFOX and FOLFIRI exert synergistic effects by combining the DNA synthesis-inhibiting property of 5-FU with the DNA-damaging properties of OX and IRI. FA potentiates the efficacy of 5-FU, upregulating malignant cell death. This broad mechanism of action suppresses chemotherapy resistance.[20] FOLFOX and FOLFIRI combinations time-dependently exerted antiproliferative on HT29 cells, significantly decreasing cell viability from 24 to 72 h of treatment. However, the viability rates of CaCo-2 cells were higher than those of HT29 cells even after prolonged treatment duration. As HT29 cells exhibit rapid division, they are highly sensitive to chemotherapeutic drugs that target cell division.[21] The incidence of multidrug resistance, which prevents the cellular entry of chemotherapeutic agents, in CaCo-2 cells was higher than that in HT29 cells. Drug resistance, a major limiting factor for cancer treatment, often develops in patients with colorectal cancer during advanced cancer treatment.[22] To overcome drug resistance, the application of a combination of chemotherapeutic agents has been proposed. The elucidation of the mechanisms of known chemotherapeutic drugs and their combinations and the factors related to chemotherapy resistance will aid in the selection of optimal treatment strategies.[23] In this preliminary study, the effects of chemotherapeutic drugs on cells with different biological activities were examined. HT29 and CaCo-2 cells isolated from patients with colon cancer are widely used *in vitro* models to evaluate the effects of potential therapeutics on colon cancer. In this study, FOLFOX and FOLFIRI combinations exerted differential growth-inhibitory effects on HT29 and CaCo-2 cells.[24] This can be attributed to the differential biological properties and resistance mechanisms of cancer cells. Consistent with the findings of this study, previous studies have demonstrated that oxaliplatin exerts growth-inhibitory effects against colon cancer cells by inducing cell death. HT29 cells were sensitive, whereas CaCo-2 cells were resistant to oxaliplatin.[25] One study reported that CaCo-2 cells developed resistance to combination treatment and that the migration ability of CaCo-2 cells was higher than that of HT29 cells after combination treatment.[26] Thus, the efficacies of FOLFOX and FOLFIRI combinations on HT29 and CaCo-2 cell lines varied depending on the biological properties of the cells.[27] In addition to FOLFOX and FOLFIRI combinations, the FOLFOXIRI combination, which is effective against metastatic colorectal cancers, has been used in various clinics worldwide. However, the application of FOLFOXIRI is limited owing to its serious side effects.[17] Hence, this study selected FOLFOX and FOLFIRI combinations due to their safety and tolerability profiles. The effects of the FOLFOXIRI combination will be evaluated in the future. In clinical practice, optimizing treatment durations and doses is critical for increasing treatment effectiveness and minimizing side effects.[28]

In conclusion, the findings of this study provide useful insights for the development of effective and targeted strategies for colon cancer. Additionally, the elucidation of the effects of drug combinations used in clinical chemotherapy will improve our understanding of the complex colorectal cancer pathogenesis and aid in developing effective targeted treatments. In addition to known chemotherapy combinations, combinatorial therapies involving targeted agents can potentially increase treatment success. Future studies must investigate the effectiveness of these combination strategies to improve the clinical outcomes of patients with colon cancer.

Ethics Committee Approval: This research utilized commercially available cell lines. As such, no ethics committee approval was necessary for this study. The research adhered to international declarations and guidelines.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – AYK, SET; Supervision – SET; Materials – AYK, SET; Data Collection and/or Processing – AYK; Analysis and/or Interpretation – AYK, SET; Writing –AYK, SET.

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