Pakistan J. Zool., pp 1-8, 2025. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240807235139

Comparison of Apoptosis Induction in Colon Cancer Cells According to Anticancer Drug Function

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Article Information
Received 07 August 2024
Revised 25 September 2024
Accepted 04 October 2024
Available online 22 January 2025 (early access)

Authors' Contribution
SHK: Conceptualization, Data curation, resources, supervision, project administration. MJO: methodology. BB and MJO: Formal analysis, writing original draft. BB and SHK: Funding acquisition. SHK and MJO: Investigation, writing review and editing.

Key words
Colorectal cancer, *Camptothecin, Rapamycin, Paclitaxel, Vinblastine*, Apoptosis

ABSTRACT

In this study, we analyzed the differences in the induction of intrinsic apoptosis by camptothecin, rapamycin, paclitaxel, and vinblastine, which are representative anticancer drugs derived from plants or chemically recombinant substances, to control colon cancer cells efficiently. The human colon cancer cell line HCT116 was used, HCT116 cells were cultured, and IC50 value of each compound was analyzed in the concentration range of 10–100 µM/mL. In addition, we analyzed the expression patterns of apoptosis markers in cells using western blotting and zymography and analyzed apoptosis activity using CCK-8 assay and Annexin V fluorescence detection. The study results showed an average IC50 value of 47.95 µM/mL. However, at different concentrations (20, 30, and 40 µM/mL) of each anticancer drug, the mechanism underlying the cell death was different. In particular, except for the camptothecin treatment group, it was con-firmed that MMP-9 activity was the mainly responsible for cell death, and camptothecin and rapamycin induced apoptosis, cytoplasmic necrosis, and morphological changes. In contrast, vinblastine and paclitaxel showed increased expression of Casp-3 and a key player in apoptosis induction, compared with the other treatment groups. Camptothecin, rapamycin, paclitaxel, and vinblastine induced apoptosis of colon cancer cells. Among them, camptothecin and rapamycin could induce cell death through various pathways compared with vinblastine and paclitaxel.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the second most prevalent cancer in South Korea (Cha and Kim, 2019; Shin *et al*., 2023). In 2021, the incidence rate of CRC in South Korea was 45 cases per 100,000 individuals, which was the highest among 184 countries. This rate is more than twice the global average (19.8 cases) and significantly surpasses that in other advanced nations, such as Slovakia (42.7 cases), Hungary (42.3 cases), and Denmark (40.5 cases) (Thursby and Juge, 2017; Kim and Lee, 2021). The medical community recognizes CRC as a highly significant cancer. Early detection is crucial for effective treatment of CRC owing to late onset of symptoms. Major risk factors associated with CRC include aging, a red meat-centered diet, obesity, smoking, alcohol consumption, and genetic predisposition (Sung *et al*., 2019; Patel *et al*., 2022). Surgical resection and postoperative chemotherapy is the standard approach for controlling the spread of cancer cells. However, chemotherapy often serves palliative purposes, and its efficacy is compromised by the development of chemoresistance to second-line treatments in patients with CRC (Shin *et al*., 2019; Kang *et al*., 2019). Chemotherapy is toxic to normal cells, leading to increased necrosis and reduced apoptosis.

These findings underscore the critical need for a strategic therapeutic approach that emphasizes the use of natural substances or appropriate anticancer agents to achieve safe and effective synergies rather than relying

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0030-9923/2025/0001-0001 $ 9.00/0

solely on cytotoxic drugs for the management of patient with CRC (Kim *et al*., 2020, 2023; Kim, 2022). Research on anticancer effects of natural compounds and other chemicals related to CRC has been steadily increasing since 1990 and is currently ongoing. Specifically, these substances reduce cellular toxicity, induce natural cell death, and regulate cytokines through immune responses in CRC cells (Huang *et al*., 2021; Abdulridha *et al*., 2020; Zhao *et al*., 2022).

Camptothecin (CPT) is a notable compound that inhibits DNA topoisomerase activity and induces cell death (Beretta *et al*., 2013). Another example is rapamycin that suppresses the activity of the mechanistic target of rapamycin (mTOR) in cells, thereby promoting autophagy and controllinsg cancer cells through self-regulation (Selvarani *et al*., 2021). Additionally, paclitaxel induces the activation of M1/M2 macrophage polarization by stimulating phosphatidylinositol 3-kinase γ (PI3Kγ), thus modulating immune responses in cancer cells (Song *et al*., 2022). Vinblastine, another natural compound known for its immune-enhancing effects and regulation of cancer cell metabolism, regulates the normal function of microtubules within cancer cells, thereby controlling cell division (Moreno-Velasco *et al*., 2022). In addition, it induces morphological changes in cancer cells through intracellular toxicity.

These approaches to cancer cell control focus not only on direct destruction of cancer cells or simultaneous cytotoxicity to surrounding cells but also on regulating intrinsic cellular processes to induce cell death. However, comparative data regarding the practical differences among the aforementioned anticancer agents are scarce, and a comprehensive comparison of pathways actively involved in inducing cell death using cancer cell lines is lacking (Zhai *et al*., 2023; Tong *et al*., 2022).

Therefore, in this study, we aimed to analyze the differences in cell death induction among camptothecin, rapamycin, paclitaxel, and vinblastine at varying concentrations using CRC cell lines.

MATERIALS AND METHODS

Culture of HCT116 cell line

Approximately 1x10^5 HCT116 cells (ATCC, Manassas, VA, USA), human CRC cell line used in this study, were cultured in 6-well plates in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 3% antibiotic (Sigma, St. Louis, MO, USA), replaced with fresh media every 48 h, and cultured for one week. IC_{50} (half-maximal inhibitory concentration) was then determined. To measure the cytotoxicity, camptothecin, rapamycin, paclitaxel, and vinblastine were used in the concentration range of 10 μM/mL to 100 μM/mL, and Ez-Cytox (Enhance Cytotoxicity Assay Kit: Dogen, Seoul, Korea) was used according to the manufacturer's instructions. Cell viability (%) was calculated as the ratio of the optical density of cells treated with each compound to that of the control group.

Cytotoxicity assay

Cytotoxicity of each compound against HCT116 cells was determined at various concentrations of 20, 30, and 40 μM/mL; the cells were cultured for 24 h, and TI2-U (Nikon, Tokyo, JAP) was used for morphological analysis of the cells. The cytotoxicity of each compound against the cancer cell line was measured by ELISA using the Cell Counting Kit-8 (CCK-8; Sigma) assay, which is based on dehydrogenase activity detection in viable cells. In this assay, WST-8 was reduced, and absorbance of the product was measured at 450 nm, according to the manufacturer's instructions. A comparative analysis was performed based on the OD values of untreated cells (Kim *et al*., 2023).

Western blot

Proteins (50 μg) extracted from HCT116 cells cultured with each compound were separated by 13% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) and blocked with 5% non-fat dry milk. The primary antibodies for β-actin (sc-47778: Santa Cruz Biotechnology Inc., Texas, USA), TNF-α receptor (sc-374186: Santa Cruz Biotechnology Inc.), and Casp-3 (ab13847: Abcam, Cambridge, UK) were mixed with blocking buffer, incubated for 2 h, and then washed thrice with washing buffer (0.1% v/v Tween 20, 50 mM Tris-HCl (pH 7.6), 200 mM NaCl) for 15 min each time. Anti-rabbit or anti-mouse secondary antibodies (Abcam) were diluted to 1:3000 in blocking buffer and incubated for 2 h. Afterward, the membrane was exposed to electrochemiluminescence detection reagent (Sigma) for 5 min and then analyzed using Kodak IS4000MM (Kodak, NY, USA) for 10–20 min.

Gelatin zymography

Total protein (50 μg) was added to 10 μL FOZ loading buffer (5% bromophenol blue, 10% SDS, and 2% glycerol) to analyze matrix metalloproteinase (MMP) response against HCT116 cell proteins. After mixing and incubation on ice for 5 min, electrophoresis was performed at 150 V for 1 h and 30 min using SDS-PAGE gel containing 100 mg/mL gelatin. After electrophoresis, the gel was incubated with renaturation buffer (2.5% Triton X-100, 1-X PBS) twice for 20 min to induce protein regeneration

and then washed with sterile water for 20 min. The cells were placed in zymography reaction buffer (1 M Tris-HCl pH 7.5, 5 M NaCl, 1 M CaCl2, 0.2 mM ZnCl2, 0.2% Triton X-100, 0.02% NaN_3) and allowed to react for 18 h at 37°C. After completion of the reaction, the zymography gel-derived proteins were stained with Coomassie Brilliant Blue (Bio-Rad) for 1 h. The faded and discolored parts were then analyzed (Kim *et al*., 2013).

Cell cycle analysis by propidium iodide (PI/ANNEXIN V)

To analyze the membrane damage of HCT116 cells after treatment with each compound, the cells were separated using 0.05% trypsin-EDTA (Thermo Fisher Scientific Inc., Waltham, USA) and then analyzed using PI/Cell Cycle Analysis Kit (Canvax Biotech, Córdoba, Spain). Fluorescence intensity of PI-red fluorescence (%) and ANNEXIN V-green fluorescence (%) was measured by repeating the red/green fluorescence signal approximately five times or more according to the rich fluorescence application method of Guava Muse flow cytometer (Millepore, Turku, Finland).

Immunofluorescence of annexin V- FITC/PI

Apoptosis and necrosis of HCT116 cells were analyzed by treating the cells with each compound; the cells were cultured in 12-well plates for 24 h, fixed with 3% paraformaldehyde, and incubated in Dulbecco's Phosphate-Buffered Saline (D-PBS; Thermo Fisher Scientific. Inc., Waltham, MA, US) and V-FITC (Shanghai Beibo Ltd, China) analyzed according to the manufacturer's instructions. The cells were then analyzed using a confocal microscope (Nikon C1; Nikon) (Zhang *et al*., 2011).

Immunofluorescence of Casp-3 and BCL-2

The protein expression of apoptosis-associated markers in HCT116 cancer cells was analyzed by immunostaining, following our previously reported protocols (Ji *et al*., 2019; Im *et al*., 2019). Cells treated with each anticancer drug were fixed in 4% paraformaldehyde overnight at 4°C, washed for 30 min in PBS, and permeabilized with 0.2% Triton X-100 for 30 min at room temperature. After blocking with 3% bovine serum albumin in PBS, the samples were incubated with antibodies against the active forms of BCl-2 (sc-492, Santa Cruz Biotechnology Inc.), Caspase-3 (sc-373730, Santa Cruz Biotechnology Inc.),), actin (sc-47778, Santa Cruz Biotechnology Inc.) and Dynactin p62 (sc-25730, Santa Cruz Biotechnology Inc.) at a 1:300 dilution. Subsequently, the samples were washed and incubated at room temperature for 2 h with secondary antibodies: Alexa Fluor™ 488 (A11001, Invitrogen, MA, USA) and Goat Anti-Rabbit IgG (H+L) Dylight 594 (35560, Invitrogen, MA, USA), both at a 1:300 dilution. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma), and the samples were mounted using fluorescence mounting medium. Finally, they were imaged using a fluorescent microscope (TI2-U: Nikon, Tokyo, Japan)

Statistical analysis

ELISA data were analyzed using the Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, version 23.0; IBM Corp, Armonk, NY). Duncan's multiple range test was used to determine the differences in mean values for each treatment group. Statistical significance was set at $P<0.05$.

RESULTS

Cell viability and IC_{50} of anticancer drug-treated HCT116 cells

The survival rate of HCT116 cells after treatment with anticancer drugs was observed to be 83–92% when exposed to 10 µM compared to 0 µM dp. However, it decreased to 21–11% at an exposure to 70 µM concentration for most anticancer drugs. Further, when exposed to a concentration of 90 µM or more, the cell survival rate rapidly decreased to 6.5–8.7%. The IC_{50} of each anticancer drug was confirmed to be 44.25 µM/mL for camptothecin, 50.65 µM/mL for rapamycin, 52.65 µM/mL for vinblastine, and 44.25 µM/mL for paclitaxel. The average IC_{50} of the added anticancer agents was 47.95 µM/mL, and it was confirmed that the added anticancer agents affected the activity of colon cancer cell lines (Fig. 1).

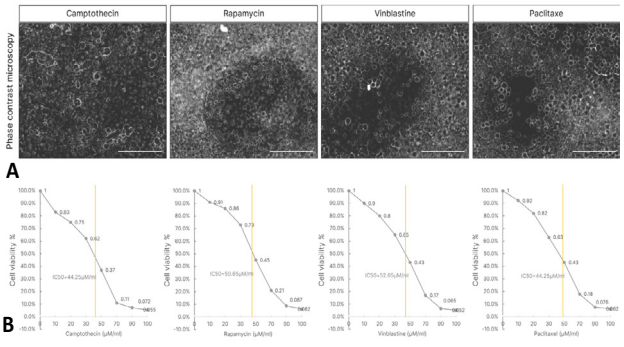

Fig. 1. Analysis of cell changes and death according to substance treatment concentration. **A**, Morphological changes in cells after treating HCT116 cells with a substance at a concentration of 40 µg/mL for 24 h. Magnification: 400X. **B**, IC50 values of anticancer drugs against colon cancer cells. IC50 values were determined in the concentration range of 10–100 µg/mL against colon cancer cells. The IC50 values were used for the competitive combination test.

Morphological changes and apoptosis in colon cancer cells exposed to anticancer drugs

The results of cells exposed to the final experimental concentrations (20, 30, and 40 μM/mL) of each anticancer drug for 24 h are shown in Figure 2. For all the drugs used in the experiment, rapid formation of lumens within cell clusters and morphological changes in the cytoplasm were observed at 40 μM/mL. Additionally, small particles and apoptotic bodies were formed around the cells. Among the drugs, camptothecin exposure led to lumen formation to a lesser extent and small particle formation to a greater extent compared with those of the other anticancer agents. For rapamycin, gradual expansion of lumens occurred with increase in its concentration from 10 μM/mL to 40 μM/mL, whereas the formation of apoptotic bodies around cell clusters occurred rapidly, compared with other drugs. Vinblastine exposure resulted in an increase in the formation of floating single cells in a concentration-dependent manner; however, there was no significant difference in cell survival between 20 μM/mL and 30 μM/mL, and cell apoptosis at 40 μM/mL was relatively low compared with other anticancer agents. Paclitaxel induced rapid morphological changes in cells at 20 μM/mL compared with other drugs, resulting in an increase in floating cell clusters and gradual formation of small particles and apoptotic bodies (Fig. 2A, E).

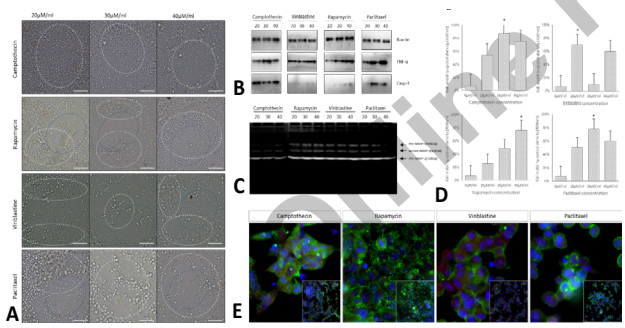

Fig. 2. Analysis of morphological changes in colon cancer cells and cell viability rate. **A**, Morphological changes in the cells depending on the type and concentration of anticancer drugs. White circles indicate morphological differences between cells, and blue circles indicate lumen formation between cell layers (Magnification: 200X). **B**, Western blot analysis of TNF-α and Casp-3 protein. **C**, The activity of MMPs was evaluated through zymography analysis in cells administered with each anticancer drug. **D**, The survival rate of cells was analyzed using the CCK-8 assay kit. **E**, The expression patterns of cytoskeletal factors actin and dynectin were confirmed using immunofluorescence analysis (Magnification: 400X). Scale bar = 100 μm. *Different letters within the same column represent a significant difference ($p < 0.05$).

Apoptosis-inducing effect of each anticancer drug at different concentrations was analyzed based on the activities of apoptosis markers TNF-α and Casp-3 (Fig. 2B, C). TNF-α activity increased in a concentration-dependent manner following treatment with all anticancer drugs, but in the case of camptothecin, it increased sharply at 30 μM/mL while it was same at 20 and 40 μM/mL. Overall, TNF-α expression increased at a higher rate in the rapamycin and paclitaxel groups than in the other treatment groups. Caps-3 expression increased in a concentration-dependent manner following treatment with camptothecin and paclitaxel, but its expression was relatively low in the rapamycin and vinblastine groups.

According to CCK-8 analysis, the apoptotic activity of camptothecin and paclitaxel followed the same pattern, whereas it was lower at 30 μM/mL than at other concentrations of vinblastine. The CCK-8 activation-based apoptotic activity of rapamycin increased with an increase in its concentration (Fig. 2D).

Analysis of the activity of MMPs involved in extracellular matrix decomposition showed that MMP-2 activity was high in all groups, but MMP-9 activity was lower in the camptothecin treatment group at all concentrations than in the other treatment groups. In contrast, the MMP-9 activity in the rapamycin group gradually increased with concentration of rapamycin, whereas it showed a constant pat-tern at all concentrations of vinblastine. However, as paclitaxel concentration increased, MMP-9 activity decreased (Fig. 2C).

Expression of apoptosis following anticancer drug treatment

Apoptotic cells significantly increased in cells treated with each anticancer drug within 48 h (Fig. 3A and Table I). The order of apoptosis cell rate from highest to lowest was Paclitaxel > Vinblastine > Camptothecin > Rapamycin. Paclitaxel significantly attenuated cells in early apoptosis compared to the different treatment groups ($p < 0.05$). The early apoptotic (LR) rate was approximately 6.81E+05 in rapamycin and 3.48E+05 in vinblastine, both significantly higher than the 3.12E+05 in camptothecin, and 3.18E+05 in paclitaxel. These results indicate that rapamycin is more effective in inducing early apoptosis than the other three anticancer drugs. The findings align with previous observations of cellular morphology.

Annexin V and PI activities were analyzed in cells following treatment with each compound at a concentration of 40 μM/mL. As a result, PI activity was high in the camptothecin and rapamycin groups, which was confirmed by Annexin V staining also. Therefore, cell death caused by the destruction of cell structure was due to necrosis and apoptosis at the same time. In contrast, the vinblastine and

Table I. Analysis of the differentially formed apoptotic cell death effect according to each anticancer treatment group.

Treatment group	Cell concentration (Cells/mL)				Gated (%)
	LL	LR	UR	UL	
Camptothecin	4.10E+05*	3.12E+05	6.95E+05**	5.66E+04*	68.36
Rapamycin	4.60E+05*	6.81E+05*	1.41E+05	5.41E+03	63.86
Vinblastine	1.84E+05	3.48E+05	6.65E+05*	3.22E+04	82.42*
Paclitaxel	9.17E+04	3.18E+05	5.04E+05	2.17E+04	87.88**

LL, Live; LR, Early apoptotic; UR, Late Apop./ Dead; UL, Debris. *,**Different letters within the same column represent a significant difference ($p < 0.05$).

paclitaxel treatment groups showed that the activity of Annexin V was more pronounced than that of PI, and indicating apoptosis as the main cause of cell death compared with the other treatment groups. In addition, cell morphological changes confirmed more obvious cytoplasmic changes compared with the other two treatment groups (Fig. 3B).

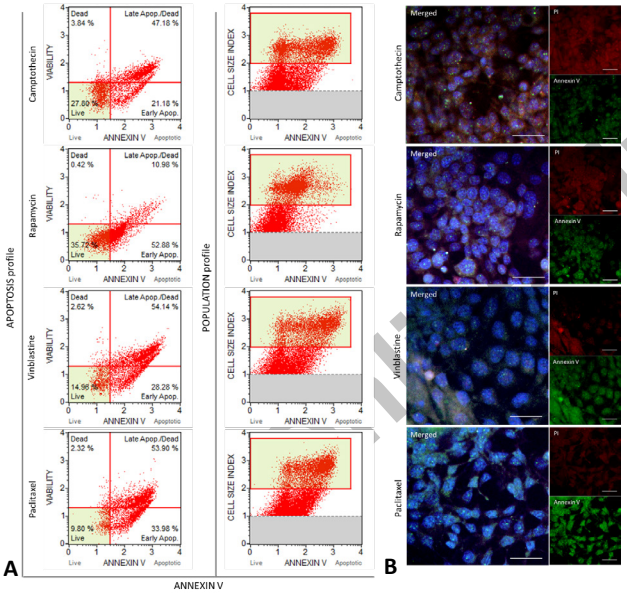

Fig. 3. Effect of an anticancer drug on apoptosis was analysed. HCT-116 cells were treated with four anticancer (40 µg/mL), respectively, stained with annexin V-FITC/ PI and analysed by flow cytometry. **A**, flow cytometry analysis. **B**, Confocal microscopy analysis of Annexin-V-FITC/PI. Magnification: 250X; Scale bar = 100 µm.

Comparison of expression of intracellular apoptosis factors and anti-apoptosis factors

The cellular expression patterns of caspase-3, a representative marker of apoptosis, and its counterpart BCL-2 were immunofluorescence analyzed (Fig. 4). Caspase-3 expression was prominent across all anticancer drugs, while BCL-2 expression was notably low. However, in the case of paclitaxel and vinblastine, which induce the highest apoptotic rates, BCL-2 expression was observed and higher than other treatment groups. Overall, the morphological changes in cell size and cell death-induced alterations in cellular morphology were consistent with the results from Figure 3, and the practical intracellular expression of caspase-3 showed a similar pattern to other findings.

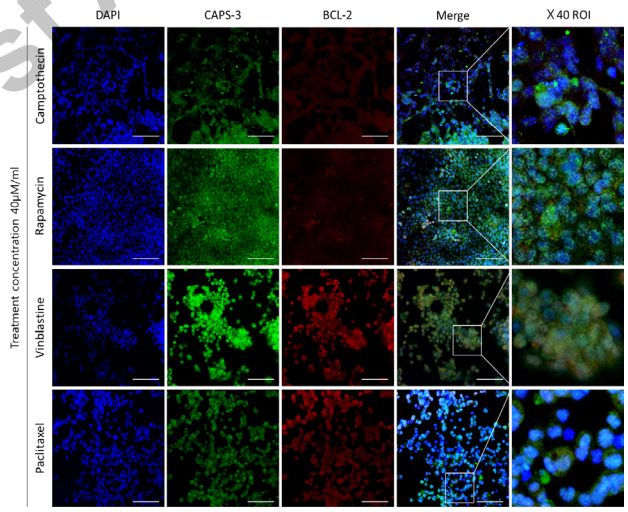

Fig. 4. Apoptosis-associated marker gene analysis of tenocytes. Immunofluorescence analysis revealed the protein expression of Casp-3 and BCL-2. The left column images depict nuclear staining with DAPI, the middle column shows the expression of Casp-3 and BCL-2, and the right column displays the overlay. These images were acquired at 200x and 400x magnification using a CCD camera attached to the Nikon microscope.

DISCUSSION

The risk of colon cancer is widely known, and many cases of colon cancer have been reported in Korea. In particular, colon cancer rapidly metastasizes, and its

prognosis is very poor even after surgery; therefore, second-line chemotherapy is required (Cha and Kim, 2019; Patel *et al*., 2022). Many studies have used natural substances as anticancer agents to control cell activity in primary cancers or induce cancer cell development and apoptosis by regulating cell death mechanisms that control the cancer cell development cycle (Shin *et al*., 2019; Kang *et al*., 2019). This study investigated the apoptotic activity of colon cancer cells using *camptothecin*, *rapamycin*, *paclitaxel*, and *vinblastine*, which control tumorigenesis, activate DNA topoisomerases, and induce apoptosis via metabolic regulation of cancer cells (Beretta *et al*., 2013; Selvarani *et al*., 2021; Song *et al*., 2022; Moreno-Velasco *et al*., 2022).

We analyzed whether the physiological activity of the colon cancer cells could be controlled. All anticancer drugs used in this study were confirmed to induce the death of colon cancer cells. In particular, camptothecsein, extracted from plants, inhibits cell division by interfering with DNA re-combination (Beretta *et al*., 2013; Rath *et al*., 2009). Similar to paclitaxel, vinblastine, also extracted from plants, inhibits cell proliferation by participating in cell division (Song *et al*., 2022; Moreno-Velasco *et al*., 2022). Rapamycin, an anticancer agent with unique characteristics, builds an autoimmune system by increasing autophagy and inhibiting mTOR activity within cells (Selvarani *et al*., 2021; Kim *et al*., 2013). Analysis of the differences in apoptosis between different compounds that control cancer cell growth in various ways revealed apparent differences in the degree of cell death and morphological changes in cells. In particular, camptothecin and rapamycin increased the formation of small particles in colon cancer cells at a concentration as low as 20 μM/mL, thereby changing the shape of the cell membrane (Beretta *et al*., 2013; Selvarani *et al*., 2021; Kim *et al*., 2013).

In contrast, vinblastine and paclitaxel lead to the formation of single cells, hindering cell growth, and formation of a suspended cell layer (Song *et al*., 2022; Moreno-Velasco *et al*., 2022). Notably, the results of this study show that camptothecin and rapamycin induce similar type of cell death at low to high concentrations through morphological changes in colon cancer cells; vinblastine and paclitaxel also induce similar type of cell death (Moreno-Velasco *et al*., 2022; Zhai *et al*., 2023). However, there was a clear differ-ence in the activities of cell death factors. Camptothecin and paclitaxel had a more sub-stantial effect on Casp-3 activity than the other anticancer drugs, while rapamycin and vinblastine showed low expression of Casp-3 (Beretta *et al*., 2013; Selvarani *et al*., 2021). The apoptotic activity of camptothe-cin and paclitaxel is likely through direct interference with cell DNA recombination to control cell division, which is consistent with existing research results (Selvarani *et al*., 2021; Zhai *et al*., 2023; Zeng *et al*., 2012).

In contrast, the anticancer effects of rapamycin and vinblastine were similar to the research results, showing that they affect cell division. However, the reactions of Annexin V and PI were different. According to previous studies, the PI response to anticancer drugs, other than rapamycin, was higher than the Annexin V response.

In this study, Annexin V and PI reactions occurred simultaneously in case of camptothecin and rapamycin, whereas vinblastine and paclitaxel were found to have a high Annexin V reaction. In conclusion, this study analyzed the differences in the mechanism of action underlying apoptosis induction by anticancer drugs with different characteristics in colon cancer cells, rather than their therapeutic effectiveness against colon cancer. Accordingly, this study showed that Camptothecin and Rapamycin are more effective than Vinblastine and Paclitaxel. The anticancer effect of these drugs is through cell morphological changes and formation of apoptotic bodies. In other words, in existing studies, camptothecin and paclitaxel showed similar results, and vinblastine and rapamycin showed similar results affecting cell metabolism.

CONCLUSION

Camptothecin, rapamycin, paclitaxel, and vinblastine induced apoptosis of colon cancer cells. In addition, the dynamic changes in cell morphology were observed for all anticancer drugs, with rapamycin and paclitaxel exhibiting higher levels of apoptotic body formation. Particle formation was more pronounced in the camptothecin and paclitaxel groups at lower concentrations than in the other treatment groups. In these results, camptothecin and rapamycin appeared to induce cell death through a sequence of morphological changes in the colon cancer cell clusters, and vinblastine and paclitaxel activated cell death by directly affecting the colon cancer cells.

Therefore, the results of this study are expected to provide primary data exploring the mechanism of anticancer drugs in the growth control of principal cancer cells, such as colorectal cancer, in the future.

DECLARATIONS

Acknowledgments

The authors express their sincere gratitude to Hankyung National University Animal Development Biotechnology Laboratory for their assistance in conducting this study.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIT) (No. RS-2023-00252042).

Statement of conflict of interest

The authors have declared no conflict of interest.

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