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Synergistic Antiproliferative Effect of Ribociclib (LEE011) and 5-Fluorouracil on Human Colorectal Cancer

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Abstract. *Background/Aim: Colorectal cancer (CRC) is one of the most common malignant tumors in the world. This study aimed to investigate the anticancer effect of the combination treatment of Ribociclib (LEE011) and 5-Fluorouracil (5-FU) on CRC cells. Materials and Methods: HT-29 and SW480 cells were treated with LEE011, 5-FU, or the combination of LEE011 and 5-FU. Cell viability and cycle were investigated through 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay and flow cytometry. The expression of cell cycle-related proteins was determined through western blot. Results: The combined treatment of LEE011 with 5-FU synergistically reduced cell viability in HT-29 and SW480 cells. Specifically, it induced cell cycle arrest at the G_1 phase, down-regulated the phosphorylation of retinoblastoma protein and the expression of p53. Conclusion: LEE011 exhibited potential as an effective therapeutic inhibitor for the combination treatment of CRC patients.*

Colorectal cancer (CRC) is one of the third-most common malignant tumors and the main reason of cancer-associated death worldwide (1). About one million cases of CRC are diagnosed worldwide each year (2). In the United States, approximately 140,000 patients were diagnosed as new CRC cases in 2019, and their estimated survival rate was 60% or less (3). In Taiwan, the prevalence, incidence, and age-related mortality of CRC are constantly increasing (4). Furthermore, 20%-25% of patients with CRC are diagnosed with metastasis; in about 25% of patients, CRC cells undergo metastasis during follow up (5). The diagnosis and survival rate of patients are highly related to tumor stage at diagnosis, and the relative survival rates are about 89% for local, 70% for regional, and 14% for distant disease (6).

Patients with CRC are generally treated with 5-fluorouracil (5-FU), a first-line treatment and the most widely used chemotherapeutic agent (7, 8). However, drug resistance in patients with CRC remains a substantial limitation of clinical treatments with 5-FU, whose efficacy is observed in only 10%-20% of patients who develop drug resistance (9, 10). Moreover, 5-FU at a therapeutic dose shows a poorly selective effect on tumors, indicating that it is highly toxic to the skin, gastrointestinal tract, and bone marrow (11). Nevertheless, the combination treatment of 5-FU with other drugs has become a considerable therapy for patients with CRC; for example, low-dose 5-FU combined with platinum can be used to manage CRC effectively (12, 13).

The dysregulation of cell cycle control is often linked to tumorigenesis and accelerated tumor growth (14, 15). Cyclin-dependent kinase-4 and kinase-6 (CDK4/6) are critical factors of cell proliferation, which bind to their protein regulator, cyclin D1, to regulate cell cycle progression (16). The cyclin D-CDK4/6-retinoblastoma protein (Rb) interaction regulates the G_1/S transition (17, 18). The function of Rb as a tumor

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suppressor protein is activated by the CDK4/6-facilitated phosphorylation of several serine and threonine residues during the G_1/S cell cycle transition (19). These cell cycle-related proteins are commonly disrupted in cancer cells to promote cell cycle progression and continued cell growth (20). The amplification of CDK4/6 and cyclin D1 are frequently encountered in breast cancers, and targeting this amplification has been identified as an effective therapeutic strategy (21). CDK4/6 have a small-molecule inhibitor called ribociclib (LEE011) developed by Novartis for the treatment of a broad spectrum of solid cancers, including breast cancer, melanoma, and neuroblastoma (22). LEE001 inhibits the phosphorylation of Rb, thereby blocking the cell cycle progression and inducing G_1 phase arrest (23). In the USA, LEE011 has been approved as a first-line treatment for advanced or metastatic breast cancer. In a study involving specific microRNA down-regulation, CDK4/6 are potential targets of therapeutic agent development to improve CRC management (24). Therefore, the potential activity of LEE011 in CRC should be further evaluated.

In the current study, the antitumor effects of 5-FU and LEE011 on the CRC cell lines HT29 and SW480 were assessed. The combination activity of LEE011 with 5-FU was evaluated to investigate the potential of LEE011 as a supplemental agent for CRC treatment. With the flow cytometry analysis, the anticancer mechanism of the combination treatment was evaluated in terms of cell cycle progression. The anticancer mechanism of LEE011 was confirmed through western blot.

Materials and Methods

Materials. 5-FU was purchased from Sigma Chemical Co. (St. Louis, MO, USA). LEE011 was provided by the Novartis Pharmaceuticals Corporation (East Hanover, NJ, USA). The colorimetric MTS assay was obtained from Promega (Madison, WI, USA). Antibodies against GAPDH and phosphorylated Rb and p53 were procured from Cell Signaling Technology (Danvers, MA, USA).

Cell culture. HT-29 and SW480 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The HT-29 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% antibiotic antimycotic solution. The SW480 cells were cultured in the Leibovitz's L-15 medium supplemented with 10% FBS and 1% antibiotic antimycotic solution. HT-29 and SW480 cells were incubated at 37˚C with a 5% CO_2 supplement.

Cell viability. HT-29 and SW480 cells were seeded at a density of 5×10^3 per well in 96-well plates overnight. The cells were treated with 5-FU and LEE011 at the indicated concentrations for 24 h. Cell viability was determined through a colorimetric MTS assay in accordance with the manufacturer's instructions. The plates were re-incubated at 37˚C for 2 h, and the absorbance of the plates was determined at 490 nm by using a Synergy 3 multimode microplate reader (BioTek, Winooski, USA).

Analysis of drug synergism. HT-29 and SW480 cells were seeded at a density of 5×10^3 per well in 96-well plates overnight. The cells were treated with 5-FU, LEE011, or their combination at the indicated concentrations for 24 h. Cell viability was determined, and the combination index (CI) values of each combination reduction were calculated using CalcuSyn2™ computer program (Biosoft, Cambridge, UK) in accordance with the method of Chou and Talalay (25). The CI was equal to 1, <1, or >1, which indicated an additive, synergistic, or antagonistic effect, respectively.

Cell cycle analysis. HT-29 and SW480 cells were seeded in a 3 cm plate at a density of 1×10^6 overnight. The cells were treated with 5-FU, LEE011, or their combination at the indicated concentrations. After 24 h of incubation, floating and adherent cells were collected through centrifugation, washed twice with ice-cold phosphate-buffer saline (PBS), and fixed with 75% ice-cold ethanol at 4˚C overnight. The cells were subsequently centrifuged to remove ethanol, and the cell pellets were resuspended in PBS containing RNase at a density of 100 μg/ml. The samples were then stained with propidium iodide at a density of 40 μg/ml in PBS at 37˚C for 30 min and analyzed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot. Cell lysates were collected with RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 2% SDS, and 1% NP-40) after 24 h of treatment. The insoluble protein was removed through centrifugation, and the concentration of soluble protein was determined using a Bio-Rad protein assay kit (Hercules, CA, USA). An equal amount of protein was analyzed through 10% SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The protein-transferred PVDF membranes were probed with specific antibodies against phospho-Rb, phosph-p53, and GAPDH. Protein signals on the membranes were developed using an ECL detection kit (PerkinElmer, CT, USA). The signal density of the target protein on the film was calculated with Quantity One (Bio-Rad, CA, USA).

Statistical analysis. Data are presented as mean±standard deviation (SD) from at least three independent experiments. Statistically significant differences between treatment and control groups were determined using Student's *t*-test, and $p<0.01$ indicated significant differences. Data were analyzed using SPSS 20.0.

Results

LEE011 and 5-FU inhibited the proliferation of colon cancer cells. The antiproliferation effects of 5-FU and LEE011 on the human CRC cell lines HT-29 and SW480 were investigated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. HT-29 and SW480 cells were treated with 5-FU or LEE011 at the indicated concentrations for 24 h. The results demonstrated that 5-FU and LEE011 exhibited a dose-dependent inhibitory effect on cell viability. LEE011 concentrations from 1 μM to 5 μM markedly inhibited the survival of HT-29 and SW480 cells (Figure 1). 5-FU was used as the positive control in the evaluation of the anticancer efficiency of LEE011. Results indicated that 1 μM

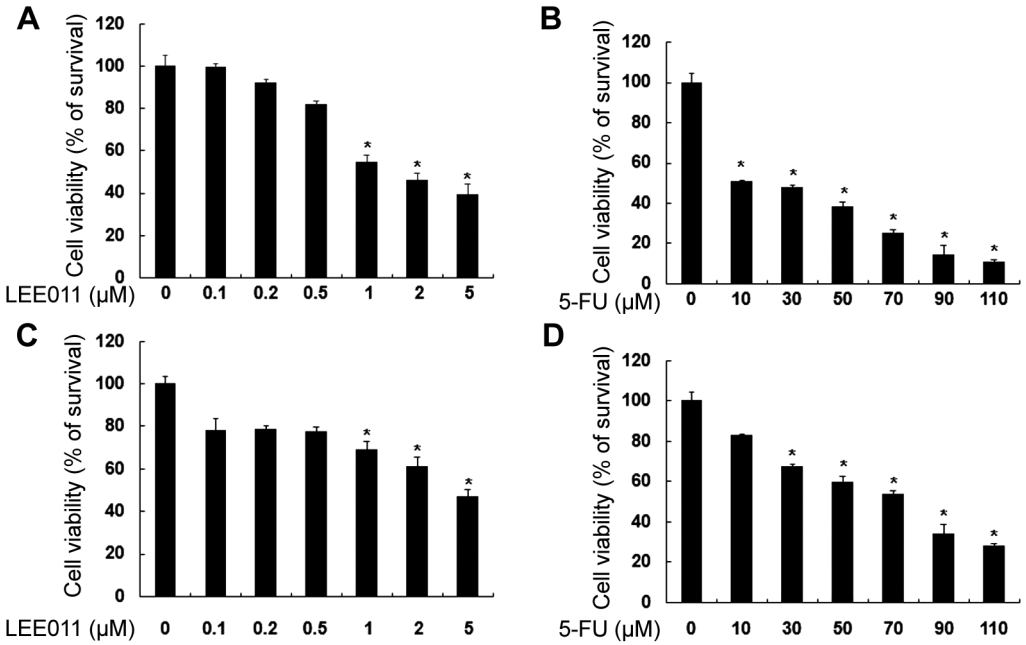

Figure 1. *Inhibitory effect of 5-FU and LEE011 on the viability of HT-29 and SW480 cells. (A, B) HT-29 and (C, D) SW480 cells were treated with 5-FU or LEE011 at the indicated concentrations for 24 h. Cell viability was determined through an MTS assay. Data were analyzed using Student's t-test and presented as mean±SD. Asterisks indicate significant differences. *p<0.01 compared with the control group.*

LEE011 exhibited the same inhibition efficiency at compared totreatment with 10 μM 5-FU in HT-29 cells (Figure 1A and B). Furthermore, 1 μM LEE011 showed similar inhibitory effect on the survival of SW480 cells to that of 30 μM 5-FU (Figure 1C and D).

LEE011 combined with 5-FU synergistically reduced the proliferation of colon cancer cells. To examine the anti-cancer activity of LEE011 in HT-29 and SW480 cells in combination with 5-FU, the cells were treated with LEE011 combined with 5-FU at the indicated concentrations for 24 h. The survival of CRC cells was also investigated by the MTS assay. The combined effects of the drugs were evaluated on the basis of CI values by using the CalcuSyn program as described in Materials and Methods. The results suggested that the CI of the combination treatment of 0.5 μM LEE011 and 30 μM 5-FU was less than 1 (0.735 in HT29 cells and 0.750 in SW480 cells) and revealed that they elicited a synergistic inhibitory effect on HT-29 and SW480 cells (Figure 2). The CI of the combination treatment of 1 μM LEE011 and 50 μM 5-FU was equal to 1, which indicated the additive inhibitory effect on HT-29 and SW480 cells.

LEE011 induced G_1 arrest and the expression of proliferation-related proteins in CRC cells. To evaluate the effect of LEE011 on the cell cycle progression of CRC cells, HT-29 and SW480 cells were treated with 0.5 μM LEE011 and 30 μM 5-FU as a positive control for 24 h. Flow cytometry results indicated that LEE011 and 5-FU treatment, respectively, caused G_1 and S phase accumulation in HT-29 and SW480 cells compared with the control group (Figure 3). The percentage of cells in the G_1 phase was 45.8% and 91.1% in HT-29 cells and 46.2% and 54.4% in SW480 cells after treatment with 0 and 0.5 μM LEE011, respectively. The percentage of cells in the S phase was 27.4% and 36.4% in HT-29 cells and 30.4% and 49.9% in SW480 cells after treatment with 0 and 30 μM 5-FU, respectively. The combination treatment of LEE011 and 5-FU mainly caused the accumulation of HT-29 and SW480 cells in the G_1 phase (Figure 3). The distribution in the G_1 phase increased from 45.8% to 61.5% in HT-29 cells and from 46.2% to 59.6% in SW480 cells. The increase in the G_1 phase was accompanied by a reduction in the proportion of CRC cells in the S and G_2 phases of the cell cycle. This result indicated that the drugs inhibited cell growth by inducing apoptosis.

To investigate the regulatory mechanism of LEE011 and 5-FU in cell cycle arrest, HT-29 and SW480 cells were treated with 0.5 μM LEE011 or 30 μM 5-FU for 24 h. The cell lysates were subjected to western blot. The results indicated that 5-FU alone and the combination treatment of LEE011 and 5-FU significantly reduced Rb phosphorylation levels. In addition, 5-FU alone, LEE011 alone, and their combination treatment

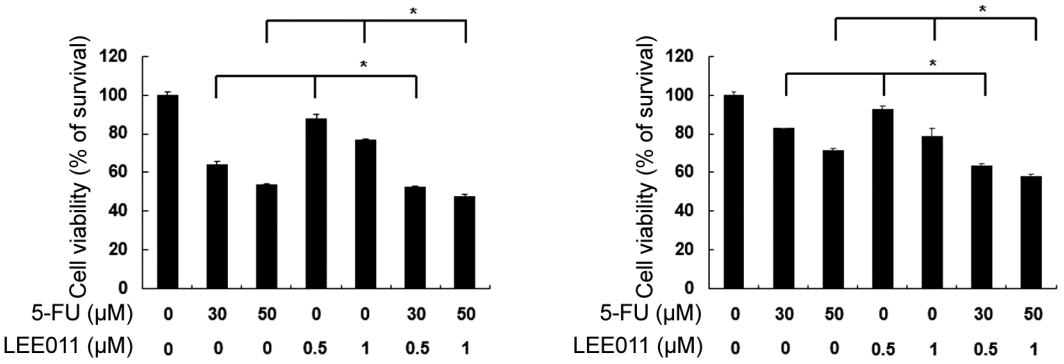

Figure 2. *Effect of the combination treatment of 5-FU and LEE011 on HT-29 and SW480 cells. (A, B) HT-29 and (C, D) SW480 cells were treated with 5-FU and LEE011 at the indicated concentrations for 24 h. The MTS assay was performed to evaluate the effect of 5-FU combined with LEE011 on the viability of HT-29 and SW480. Data were analyzed using Student's t-test and presented as mean±SD. Asterisks indicate significant differences. *p<0.01 compared with the control or single inhibitor-treated group.*

significantly induced p53 phosphorylation in HT-29 and SW480 cells compared with the control group (Figure 4). Therefore, Rb and p53 were key factors that participated in LEE011- and 5-FU-induced cell cycle arrest and diminished CRC cell growth.

Discussion

About 45% of patients with advanced stages CRC die because of a ubiquitous malignancy (2, 26). When patients with CRC are diagnosed with advanced-stage tumor and required to undergo chemotherapy, 5-FU is used as the first-line chemotherapeutic agent as it shows a constant inhibitory effect on tumor growth and displays low toxicity (27). However, 5-FU has side effects, including myelosuppression and gastrointestinal toxicity, which remain serious and common issues for patients with CRC (28). Furthermore, 50% of patients diagnosed with CRC and treated with 5-FU develop drug resistance, but its mechanism has yet to be elucidated and evaluated to establish proper treatments (29). Nevertheless, a combination treatment of 5-FU-resistant patients with advanced CRC has become an important therapeutic strategy (30). In the present study, LEE011 not only reduced the viability of CRC cells in a dose-dependent manner but also exhibited additive and synergistic effects when combined with 5-FU. These results illustrated the sensitization effect of LEE011 on the anticancer activity of 5-FU in HT-29 and SW480 cells, which indicated the potential of LEE011 as a therapeutic agent for combination treatments.

The mechanism of the anti-cancer activity of 5-FU involves its incorporation into DNA and RNA or inhibition of thymidylate synthase, causing DNA damage and cytotoxic effects, which trigger p53 activation (31). p53 phosphorylation is necessary for the maintenance of genomic stability by activating G_1, S, and G_2 checkpoints upon stress (31). In the present study, flow cytometry results indicated that 5-FU reduced the viability of HT-29 and SW480 cells by inducing S phase arrest. Western blot results confirmed that phosphorylation of p53 was induced by 5-FU in CRC cells. However, the details about the expression of regulatory proteins, such as p21, CDK4, and CDK2, related to 5-FU-induced S phase arrest in HT-29 and SW480 cells should be investigated.

LEE011 is considered a new-generation therapeutic agent against cancer cell growth, but its activity in CRC cells is still unclear. When HT-29 and SW480 cells were treated with different doses of LEE011, their viability was significantly reduced by inducing G_1 phase arrest. LEE011-induced G_1 phase arrest contributed to its synergistic effect with 5-FU against CRC cell proliferation. Upon treatment of HT-29 and SW480 cells with LEE011 alone or in combination with 5-FU, the expression of phosphorylated Rb was significantly reduced. A previous study had revealed that dephosphorylated Rb was cleaved by caspases during apoptosis but activated Rb was a poor target of caspases (32). Furthermore, the combination treatment promoted apoptosis by inducing a higher expression levels of phosphorylated p53 than that caused by LEE011 or 5-FU alone in SW480 cells. In HT-29 cells, LEE011 also significantly induced the expression of phosphorylated p53. However, the regulatory mechanism of LEE-induced cell apoptosis *via* the induction of phosphorylated p53 should be further investigated.

In conclusion, our results indicated that LEE011 has an anti-cancer effect in CRC cells, and its basic anticancer mechanism involves G_1 phase arrest induced by the down-regulation of phospho-Rb expression. As a clinically used agent for breast cancer, LEE011 exhibited a high therapeutic

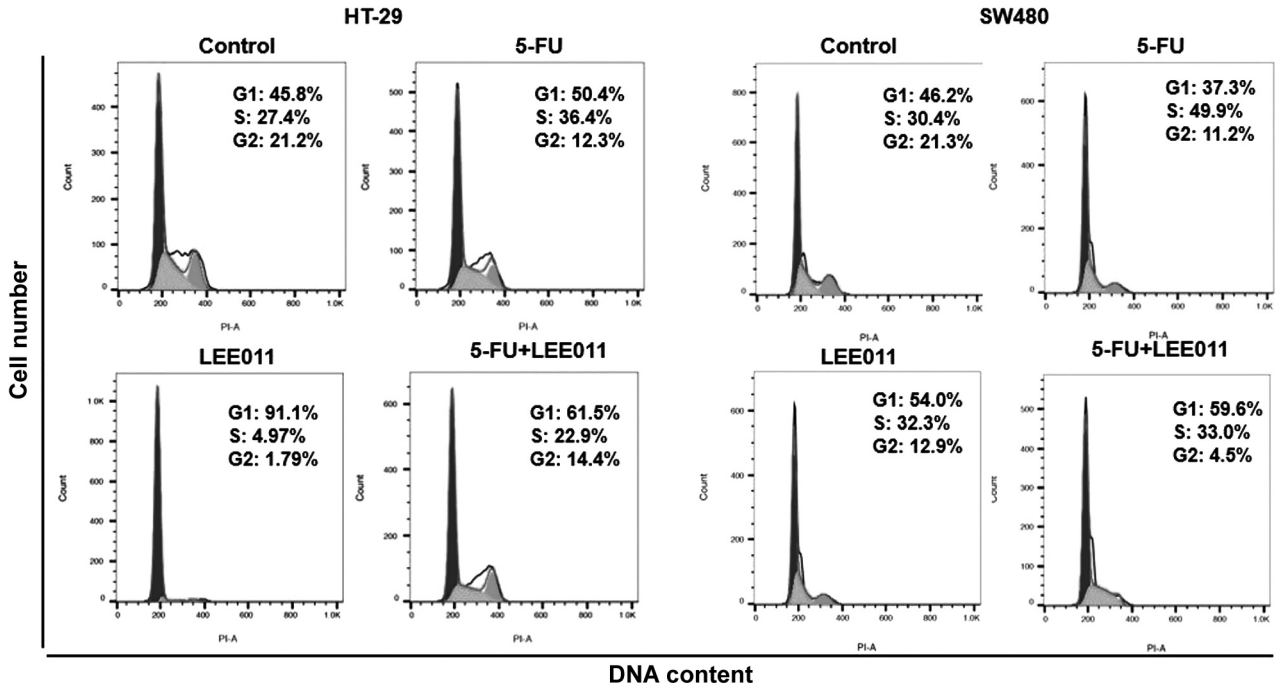

Figure 3. *Flow cytometric analysis of the effect of 5-FU and LEE011 on the cell cycle. HT-29 and SW480 cells were treated with 5-FU and LEE011 at the indicated concentrations for 24 h. They were then harvested, and the cell cycle was analyzed with propidium iodide staining. Data correspond to the percentages of cells in G_1, S, and G_2 phases.*

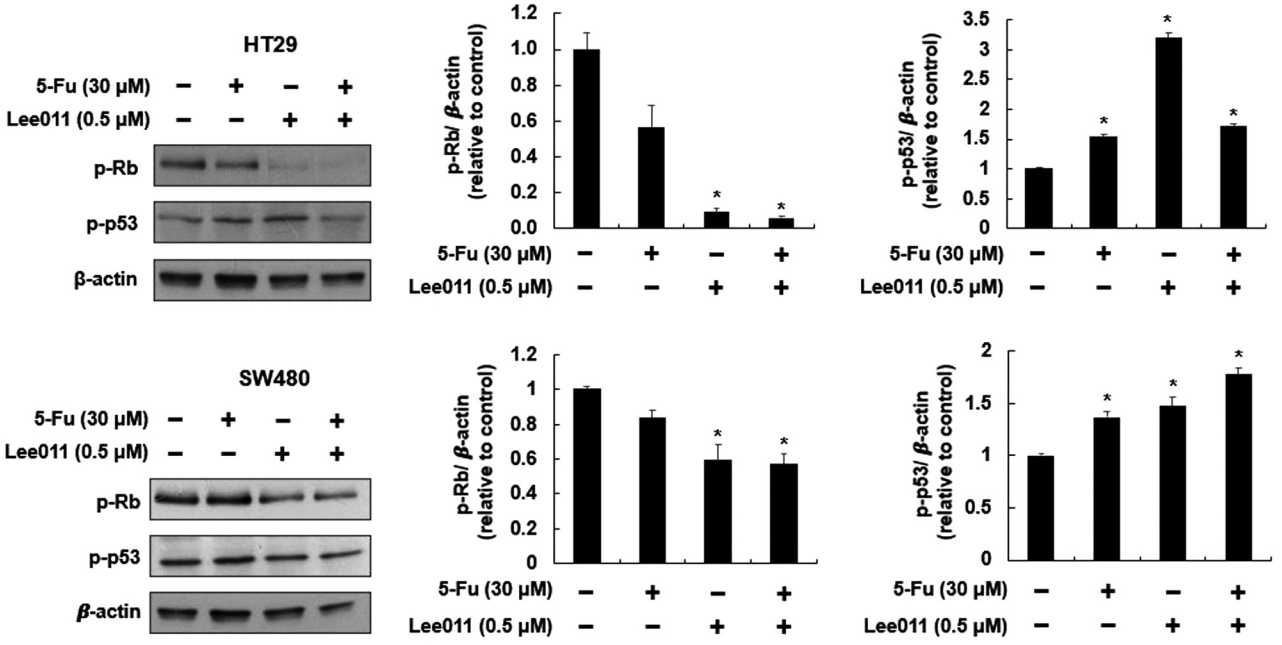

Figure 4. *Effect of 5-FU and LEE011 on the expression of cell cycle-related proteins. HT-29 and SW480 cells were treated with 5-FU and LEE011 at the indicated concentrations for 24 h. Cell lysates were collected and analyzed via western blot. β-actin served as the loading control. Data were analyzed using Student's t-test and presented as mean±SD. Asterisks indicate significant differences. *p<0.01 compared with the control group.*

potential for the treatment of patients with CRC. Further studies should be conducted to evaluate LEE011 activities in animal models of CRC.

Conflicts of Interest

The Authors have declared that no competing interests exist in relation to this study.

Authors' Contributions

Conceived and designed the experiments: YCS. Performed the experiments and data analysis: PML, HML, CIH, TST, JHC, and CIC. Wrote the paper: PML and YCS. All Authors reviewed the manuscript.

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