The Synergistic Effect of Rapamycin Combined with 5-Fluorouracil in BALB/cByJNarl Mice Bearing CT-26 Tumor Cells

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Abstract. Background: The aim of this study was to investigate the antitumor effect of rapamycin, an inhibitor of mammalian target of rapamycin (mTOR) signaling, combined with 5-fluorouracil treatment on CT-26 colorectal adenocarcinoma cells implanted into BALB/c mice. Materials and Methods: Two experiments were carried out: treatment from day 1 after CT-26 cell implantation; and treatment from day 7 after CT-26 cell implantation after the detection of a tumor mass. There were four groups in each experiment: control; treatment with 5-fluorouracil; with rapamycin; and with rapamycin with 5-fluorouracil. Results: Rapamycin combined with 5-fluorouracil significantly reduced tumor size, suppressed expression of B-cell lymphoma 2, increased tumor apoptosis, and inhibited mTOR signaling activity by de-phosphorylation of S6K. Conclusion: The results strongly suggest that rapamycin might increase the chemosensitization of tumor cells. Rapamycin combined with 5-fluorouracil treatment had a synergistic tumor-inhibition effect. Future research on rapamycin is required to develop new therapeutic strategies.

Colorectal cancer is one of the most common types of cancer worldwide (1, 2). Several treatment approaches have been used in treating colorectal cancer, including surgery, chemotherapy and radiotherapy. In advanced colon cancer, new anticancer drugs such as cetuximab (3) and bevacizumab (4) combined with chemotherapy have now become mainstay of treatment. However, the prognosis remains poor. Many studies have focused on the mammalian target of rapamycin (mTOR) signaling pathway as being a potential target of therapy for cancer (5, 6). mTOR, a member of the phosphoinositide 3-kinase-related family of signaling elements, has been reported to play a central role in controlling cancer cell growth (6-8). Rapamycin, a macrolide ester first isolated from the bacterium Streptomyces hygroscopicus (9), has been reported to lead to G1 arrest in tumor cell lines by inhibition of the mTOR pathway (10, 11).

Recent research has shown that inhibition of mTOR signaling by rapamycin can synergize with chemotherapeutic drugs in inducing apoptosis of breast, ovary and liver cancer cells (11, 12). Therefore, inhibition of mTOR signaling may augment the efficacy of chemotherapy in colon cancer, and may lead to the development of new therapeutic strategies. In the present study, we designed two different experiments to investigate the therapeutic effect of rapamycin combined with 5-fluorouracil treatment on CT-26 colorectal adenocarcinoma cells implanted into BALB/c mice.

Materials and Methods

Animals and tumor cell line. This study was approved by the local Institutional Review Board of National Chung Hsing University, Taiwan (NCHU IACUC approved number 98-84). Six-week-old male BALB/cByJNarl mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. Two to three mice were housed in a cage and provided with sterilized food and water. The mice were maintained at relative humidity of 55±5% and a constant temperature (22±2°C) with a 12-h/12-h light/dark cycle. In total, 68 mice were used in this study. All animal care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals at National Chung Hsing University.

A CT-26 murine colon carcinoma cell line (ATCC CRL-2638) was purchased from the Biosource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

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3329
**Tumor inoculation and treatment.** Two different experiments were designed: treatment initiated on the day of CT-26 cell implantation (day 1; early treatment); treatment from day 7 after CT-26 cell implantation when a tumor mass had been detected (late treatment). After the mice were anesthetized by intraperitoneal ketamine injection, a 100 μl cell suspension containing 1×10⁶ viable CT-26 cells was injected subcutaneously in the posterior leg. In experiment 1, on day 1, none of the mice had a palpable tumor, and they were randomly divided into four groups (10 in each group). In experiment 2, on day 7, all of the mice had developed a palpable tumor, and they were also divided into four groups (7 in each group). Group I in each experiment was given reverse osmosis water daily via gastric lavage as the control group; group II was injected with 5-fluorouracil intraperitoneally (100 mg/kg/week) Fluorouracil (VALEANT Valeant Pharmaceuticals, Basingstoke, UK); group III was given rapamycin (2 mg/kg/day) (Rapamycin; Wyeth Radnor, Pennsylvania, USA) via gastric lavage; and group IV was given rapamycin (2 mg/kg/day) via gastric lavage and intraperitoneal injections of 5-fluorouracil (100 mg/kg/week).

**Tumor growth was monitored every three days by measuring the greatest and the least diameters. Tumor volume was calculated using the formula: V=0.5×a×b², where a is the greatest diameter, and b is the least diameter.**

**Clinical observations and analysis.** The mice were observed daily for clinical signs, and bodyweight was measured every three days. Blood was collected for hematological examinations and hepatic and renal function tests under anesthesia at the end of the treatment period. The mice were sacrificed on day 21. The tumor specimens were divided into two parts, one of which was fixed with 10% formalin and embedded with paraffin, then assessed by hematoxylin and eosin staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (APO-BrdU™ TUNEL Assay Kit, BD-Pharmingen, San Diego, CA, USA), and the other was first preserved at −80°C and then examined for proliferating cell nuclear antigen (PCNA), B-cell lymphoma 2 (Bcl2) and vascular endothelial cell growth factor (VEGF) by western blotting.

**Statistical analysis.** All statistical analyses were performed with SAS (version 9.3; SAS Institute, Cary, NC, USA) for Student’s t-test. All data are expressed as mean±SE. p-Values less than 0.05 were considered to be significant.

**Results**

**Rapamycin slowed tumor growth.** In the early-treatment experiment, the tumor volume was reduced in the groups treated with 5-fluorouracil-, or rapamycin-alone; however, after 17 days, the tumor volume increased rapidly, and rapamycin-alone failed to control this growth (Figure 1A and C). In the late treatment experiment, rapamycin-alone did not inhibit tumor growth; however the tumor volume was reduced in the 5-fluorouracil- and rapamycin plus 5-fluorouracil-treated groups compared to the control group (Figure 1B and D). The treatment significantly reduced the tumor size in the 5-fluorouracil- and rapamycin plus 5-fluorouracil-treated groups at 9, 12, 15, 18 and 21 days compared to control mice (Figure 1D).

In the early-treatment experiment, the tumor growth rate was inhibited by 48% in the group treated by rapamycin alone, by 34% in the 5-fluorouracil-treated group, and by 60% in the group treated with rapamycin plus 5-fluorouracil compared to the control mice (Table I). In the late-treatment experiment, there was no significant difference in tumor growth in the rapamycin-treated group, however, the growth rate was inhibited by 22% in the 5-fluorouracil-treated group, and by 48% in the rapamycin plus 5-fluorouracil-treated group compared to the control mice (Table I).

**Rapamycin inhibited tumor growth.** In the late-treatment experiment, hematoxylin and eosin staining showed tumor cell shrinkage and death in the treatment groups, but tumor cell shrinkage and death in the treatment groups, and in particular, the group treated with 5-fluorouracil combined with rapamycin (Figure 2A).

**Rapamycin increased the number of TUNEL-positive cells.** In the early- and late-treatment experiment, TUNEL assay revealed that rapamycin alone and 5-fluorouracil alone increased the number of TUNEL-positive cells. However, there was a significant increase in the number of TUNEL-positive cells in the group treated with 5-fluorouracil combined with rapamycin (Figure 2B).

**Rapamycin inhibited PCNA expression.** In the early- and late-treatment experiment, western blotting showed that tumor proliferation and mitosis were suppressed in the group treated with 5-fluorouracil combined with rapamycin. Rapamycin or 5-fluorouracil alone showed a trend for inhibition of PCNA expression but not significantly (Figure 2C).

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<th>Table I. Tumor weight in the BALB/cByJNarl mice after sacrifice in the early treatment experiment.</th>
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<td>Tumor weight (g)</td>
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<td>Early treatment</td>
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<tr>
<td>Control</td>
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<tr>
<td>5-FU</td>
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<td>Late treatment</td>
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*p<0.05; ***p<0.001 vs. control. Tumor inhibition rate (%)=(C-T)/C×100%, where C: tumor weight of control group, and T: tumor weight of treatment group. RAPA: Rapamycin; 5-FU: 5-fluorouracil.
Rapamycin suppressed of Bcl2 expression. In the early- and late-treatment experiments, 5-fluorouracil combined with rapamycin significantly suppressed Bcl2 expression as compared with the control group (Figure 3A).

Rapamycin inhibited VEGF expression and led to dephosphorylation of S6K. In the early-treatment experiment, western blotting showed that VEGF expression was significantly inhibited in the group treated with rapamycin combined with 5-fluorouracil. But in the late-treatment group, VEGF expression was slightly inhibited in the combination-treated group (Figure 2D). In the late treatment experiment, western blotting showed that rapamycin increased dephosphorylation of S6K (Figure 3B).

Taken together, the results show that rapamycin combined with 5-fluorouracil slowed tumor growth, slightly suppressed VEGF expression, increased tumor cell apoptosis, significantly suppressed Bcl2 expression, and dephosphorylated S6K.

Discussion

In this study, our results show that rapamycin alone transiently delayed tumor growth in early treatment but failed to control tumor growth in late treatment. Treatment with rapamycin combined with 5-fluorouracil significantly reduced tumor size, suppressed Bcl2 expression and increased dephosphorylation of S6K (Figure 3).

The prognosis for patients with clinically advanced stage colorectal cancer after chemotherapeutic treatment remains poor. The regimens for colorectal cancer are primarily based on the pyrimidine analog 5-fluorouracil (13). However, recent studies have shown that rapamycin can enhance chemotherapy-induced cytotoxicity (12, 14). Rapamycin, a mTOR inhibitor, has been tested in vivo and in vitro (6, 15), and it has been shown to be a critical effector in cell-signaling pathways in regulating human cancer (16, 17). Therefore, we designed this study to investigate the therapeutic effect of rapamycin combined with 5-fluorouracil treatment on CT-26 colorectal

Figure 1. Images of tumors showing relative size after sacrifice in the early (A) and late (B) treatment experiments. Tumor size of BALB/cByJNarl mice after early (C) and late (D) treatment. Early treatment: rapamycin (RAPA) treatment started directly on day 1 of the CT-26 tumor cell injection. Late treatment: RAPA treatment started when tumors were detected at day 7. *p<0.05, **p<0.01, ***p<0.001 vs. control; ##p<0.01 vs. 5-fluorouracil (5-FU).
adenocarcinoma cells implanted into BALB/c mice with two major aims: to determine whether rapamycin treatment could inhibit the mTOR pathway; and whether rapamycin combined with 5-fluorouracil would have a synergistic effect.

Some studies reported that mammalian target of rapamycin complex 1 (mTORC1) is sensitive to rapamycin whereas mTORC2 is resistant to rapamycin. mTORC1 regulates activity via two important downstream effectors, the ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E) (18-20). Rapamycin has been reported to cause dephosphorylation of all sites in S6K1 (21), regulate mTORC1 activity (16), and inhibit tumor growth and G1 phase progression (22). Therefore, our first hypothesis was that rapamycin could inhibit the mTOR pathway by directly inhibiting tumor proliferation and apoptosis.

Our results show that rapamycin indeed slowed tumor growth in the early-treatment experiment with rapamycin by about 48% compared with the control group. When rapamycin was combined with 5-fluorouracil, the inhibition rate increased to 60%. Rapamycin alone caused a delay in tumor development during the first 17 days, after which the tumor began to grow rapidly (Table I, Figure 1A and C). In the late-treatment experiment, rapamycin alone failed to control tumor growth, however the tumor size significantly decreased in the combination group, with an inhibition rate of 48% compared to the control mice (Table I, Figure 1B and D).

Hypoxia is known to activate mTOR signaling, thereby enhancing angiogenesis (23). The inhibition of mTOR signaling may thus be the key to the anti-angiogenesis effect. In rapamycin-sensitive mTORC1, deactivation of S6K occurs

Figure 2. A: Histopathology of tumor mass cells in the BALB/cByJNarl mice in the late treatment experiment (treatment initiated 7 days after the implantation of the cancer cells and when a tumor had been detected). i: Tumor cell necrosis; ii: surviving tumor cell. Hematoxylin and eosin stain (×100). TUNEL-positive cells (B), and investigate western blot analyses of proliferating cell nuclear antigen (PCNA) (C) and vascular endothelial cell growth factor (VEGF) (D) expression in the tumor mass of BALB/cByJNarl mice. i: Early treatment: rapamycin (RAPA) treatment start directly on day 1 of CT-26 tumor cell injection. ii: Late treatment: RAPA treatment started when tumors were detected at day 7. *p<0.05, **p<0.01 compared to control group.
**Figure 3.** Western blot analysis of B-cell lymphoma-2 (Bcl2) (A) and phosphorylation S6 kinase (pS6K) (B) expression in the tumor mass of BALB/cByJNarl mice. 

i: Early treatment: rapamycin (RAPA) treatment start directly on day 1 of CT-26 tumor cell injection. 

ii: Late treatment: RAPA treatment started when tumors were detected at day 7. *p<0.05, **p<0.01 compared to control group.

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**Figure 4.** A working model of the synergy between rapamycin and 5-fluorouracil (5-FU). Rapamycin combined with 5-FU therapy suppressed B-cell lymphoma 2 (Bcl2) expression, and might inhibited mTOR signaling activity by dephosphorylation of ribosomal S6 kinase (S6K), with a synergistic tumor inhibitory effect on cancer cells.
early and transiently to hypoxia. However, rapamycin-resistant mTORC2 signaling may also play an important role associated with hypoxia and altered angiogenesis (24).

In order to test this hypothesis, we analyzed treatment effects by the TUNEL assay, PCNA and Bcl2 staining and western blotting. The results show that rapamycin alone only slightly inhibited tumor proliferation and mitosis without a significant difference (Figure 2B and C, and 3A).

In western blot analysis of VEGF and p-S6K expression, the early treatment, VEGF expression was significantly inhibited in the group treated with rapamycin combined with 5-fluorouracil, but in the late treatment group, VEGF expression was slightly inhibited in the combination-treated group (Figure 2D), rapamycin increased dephosphorylation of S6K In the late treatment group. (Figure 3B)

We found that rapamycin transiently delayed tumor growth in the early stage when a tumor had not been detected, whereas after the development of a tumor, rapamycin alone failed to control tumor growth. These findings suggest that rapamycin cannot directly inhibit tumor proliferation and apoptosis.

Recent studies have shown synergistic antiproliferative effects of mTOR inhibitors in combination with 5-fluorouracil in scirrhous gastric cancer, and hepatocarcinoma cells (12). Phosphoinositide 3-kinase/mTOR inhibitors may combine better with 5-fluorouracil due to cellular heterogeneity in sensitivity their inhibition in mutant gastric cancer cells (25). It is also possible that rapamycin combined with 5-fluorouracil had a synergistic.

The proteins of the Bcl2 family are key regulators of programmed cell death (26, 27). Overexpression of Bcl2 protein may be linked to chemoresistance and tumorigenesis (28-30). Several studies have shown mTOR kinase damages microtubules and inactivates Bcl2 proteins (31-33). mTOR is a kinase that lies directly in regulation of p70/S6K, and regulation of Bcl2 phosphorylation in the mTOR and Bcl2 pathway has been reported (5, 33). The results of this study showed that rapamycin combined with 5-fluorouracil significantly suppressed Bcl2 expression, and dephosphorylated S6K, synergistically suppressing tumor proliferation and mitosis. A working model to explain this is shown in Figure 4.

In this study, we found that rapamycin combined with 5-fluorouracil had a strong synergistic effect on tumor cell inhibition. Rapamycin might inhibit the mTOR/Bcl2 pathway and increase chemosensitization of tumor cells. This might be exploited after more in vivo and in vitro studies to develop new therapeutic strategies for colon cancer.

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References


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