

Blockage of 2-Deoxy-D-Ribose-Induced Angiogenesis with Rapamycin Counteracts a Thymidine Phosphorylase-Based Escape Mechanism Available for Colon Cancer under 5-Fluorouracil Therapy

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ABSTRACT

Purpose: Colorectal neoplasms remain a leading cause of cancer-related deaths. A recognized weakness of conventional 5-fluorouracil (5-FU) therapy relates to expression of the intracellular enzyme, thymidine phosphorylase (TP). Although TP promotes 5-FU cytotoxicity, TP-derived 2-deoxy-D-ribose (dRib) counterproductively stimulates tumor angiogenesis. Here, the newly discovered antiangiogenic drug rapamycin was combined with 5-FU to counteract the potential escape mechanism of dRib-induced angiogenesis.

Experimental Design: Orthotopic tumor growth was assessed in rapamycin and 5-FU-treated BALB/c mice with TP-expressing CT-26 colon adenocarcinoma cells. To examine liver metastasis, green-fluorescent protein-transfected CT-26 cells were visualized by fluorescence microscopy after intraportal injection. Cell counting and Ki67 staining were used to determine *in vitro* and *in vivo* cell expansion, respectively. *In vitro* angiogenic effects of dRib were assessed with endothelial cell migration and aortic ring assays. Western blotting detected dRib effects on p70/S6 kinase activation.

Results: Rapamycin treatment of mice bearing orthotopic tumors inhibited tumor growth more than did 5-FU, and mice treated with both drugs typically developed no tumors. In the liver metastasis assay, combination therapy blocked metastatic expansion of solitary tumor cells. Inter-

estingly, complex drug activities were suggested by tumor-cell proliferation being more sensitive to 5-FU than to rapamycin *in vitro*, but more sensitive to rapamycin *in vivo*. With regard to angiogenesis, dRib-induced endothelial cell migration and aortic ring formation were completely abrogated by rapamycin, correlating with blockage of dRib-induced p70/S6 kinase activation in endothelial cells.

Conclusions: Inhibition of dRib-induced angiogenesis with rapamycin counteracts a potential TP-based escape mechanism for colorectal cancer under 5-FU therapy, introducing a novel, clinically feasible, combination treatment option for this common neoplasm.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related deaths among North Americans (1). Although the incidence of local recurrence after surgical resection has been lowered considerably with the use of radical surgical techniques and adjuvant chemotherapy regimens, 40% of patients with nodal-positive disease die from tumor progression (2). The most frequent cause for this high mortality rate after surgery is metastasis of the primary tumor to the liver. Therefore, preventing the redevelopment of metastatic cancer from residual tumor cells is a treatment goal.

At present, regimens for the adjuvant treatment or palliation of recurrent and metastatic colorectal cancer are primarily based on the pyrimidine analog 5-fluorouracil (5-FU) or its derivatives. In a palliative setting, 5-FU-based chemotherapy has improved overall survival, but a high percentage of patients continue to die from metastatic cancer (2). Recently, expression of the enzyme thymidine phosphorylase (TP) on tumor cells and stroma has been correlated with poor survival and low response to 5-FU-based chemotherapy regimens in colon cancer and in other malignancies (3–5). The interesting conflict observed in treating TP-expressing cancer is that, in one respect, TP is a key enzyme in the metabolic conversion of 5-FU to its active cytotoxic form, 5-fluoro-dUMP, thus enhancing 5-FU cytotoxicity (6, 7), but, in another respect, its enzymatic activity promotes tumor angiogenesis *in vivo* (8–10), correlating with increased microvessel density in human colon cancer (11). TP exerts its main angiogenic effects by enzymatic conversion of thymidine into thymine and 2-deoxy-D-ribose-1-phosphate, which is dephosphorylated into the proangiogenic molecule, 2-deoxy-D-ribose (dRib; Ref. 12). Indeed, TP stimulation of *in vivo* angiogenesis can be negated by inhibiting dRib with the stereoisomer 2-deoxy-L-ribose (13). Other studies confirm that dRib accounts for the proangiogenic effects of TP (14, 15), but the mechanism(s) are not yet established. Interestingly, under 5-FU treatment, dRib-induced angiogenesis in TP-expressing tumors may counteract the positive cytotoxic effects of 5-fluoro-dUMP. This hypothesis correlates with the observation that tumor cells with

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strong expression of TP *in vitro* are highly sensitive to 5-FU cytotoxicity (6, 7); however, *in vivo* where angiogenesis plays a critical role, high TP expression in tumors can signal a poor prognosis with different types of cancer (8, 9), including colorectal cancer (3).

On the basis of this background, and recent clinical data indicating the effectiveness of antiangiogenic therapy against colorectal cancer metastasis (16), we sought a drug to combine with 5-FU, with the intention of inhibiting a potential TP-dRib-promoted angiogenesis escape mechanism. For this purpose, we chose the newly discovered antiangiogenic substance, rapamycin (17). Besides the fact that rapamycin has inhibitory effects on vascular endothelial cell growth factor (VEGF)-mediated endothelial cell signaling, which could interfere with the proangiogenic activity of dRib (17), it was chosen because of its proven safety record in humans when used long-term to prevent transplant rejection (18). Here we report a series of experiments in a mouse TP-expressing colon adenocarcinoma model showing that addition of the antiangiogenic drug rapamycin to 5-FU cytotoxic therapy prevents early orthotopic tumor growth and inhibits the emergence of hepatically seeded colon cancer cells into liver metastases. Furthermore, we show that dRib stimulation of angiogenesis is at least partially due to activation of the intracellular signaling molecule p70/S6 kinase, which is known to promote endothelial cell growth downstream of the rapamycin-sensitive molecule, mammalian target of rapamycin (mTOR; 19, 20). Consistent with this finding, we show that rapamycin does indeed block dRib-induced angiogenesis *in vitro*. These data provide the basis for a novel mechanistic approach to control locally recurrent or metastatic colon adenocarcinoma.

MATERIALS AND METHODS

Mice and Tumor Cell Lines. Male 20–25g BALB/c and BALB/c severe combined immunodeficient mice (Harlan Winkelmann, Borcheln, Germany) were used. Animal procedures were approved by the regional authorities.

CT-26 cells used in our experiments were derived from a murine BALB/c colon adenocarcinoma (21). Tumor cells were maintained by cell culture in RPMI 1640 supplemented with 10% fetal bovine serum. To track the same tumor cells *in vivo* in metastasis-assay experiments, we transfected CT-26 cells to stably express green-fluorescent protein (GFP; cells provided by Prof. Werner Falk, University of Regensburg, Regensburg, Germany), as we have described previously (22).

Orthotopic and Metastatic Tumor Models. For orthotopic tumor implantation, 1×10^6 unaltered CT-26 cells were injected subserosally into the cecum of syngeneic, immunocompetent, BALB/c mice, as described previously (21). Untreated tumors in this model typically lead to intestinal blockage starting at 2 weeks; therefore, for direct comparisons with treatment groups, mice were normally sacrificed 14 days after CT-26-cell injection, and tumors were weighed. All of the mice were also examined for local lymph node and liver metastases. Furthermore, enlarged lymph nodes or liver tissue were processed for H&E staining to confirm the presence of tumor.

To observe the development of colon metastases in the liver, 3×10^5 GFP-expressing CT-26 tumor cells were injected

directly into a branch of the portal vein at the distal cecum of BALB/c severe combined immunodeficient mice and were tracked, as described previously (22). Also included in the injection mixture were 10- μ m red-fluorescent microspheres (Fluorospheres; Molecular Probes, Leiden, the Netherlands), at a ratio of 10 tumor cells:1 microsphere. The microspheres serve as permanent markers to verify successful tumor-cell injection and to numerically account for tumor foci, as described previously (22, 23). Ten days after tumor-cell inoculation, mice were sacrificed, and the surface of the left liver lobe was directly examined and photographed by fluorescence microscopy (Axiotech Vario; Zeiss, Göttingen, Germany). Thirty microscopic fields ($\times 200$) from the liver surface were examined for tumor cells, and identifiable tumor entities were classified into one of four categories: (a) a single cell; (b) a multicellular focus (2–12 cells); (c) a small metastasis ($\leq 200 \mu\text{m}$); or (d) a large metastasis ($> 200 \mu\text{m}$). Quantitation was performed by also counting red fluorescent beads per field and calculating the number of each tumor entity (per 1000 cells injected) from the number and ratio of cells:beads injected. In some experiments, the entire cut surface of three 2-mm slices of the liver lobe was then examined at low magnification to determine the hepatic replacement area (ratio of tumor area:total liver area). These images were recorded by a video camera (modified Sony 3CCD Color Video Camera; AVT Horn, Aalen, Germany) on super-VHS tapes for later off-line analysis. Adjacent sections of liver tissue were also processed for standard H&E staining.

Rapamycin and 5-FU Treatment in Mice. Rapamycin (Wyeth Pharma GmbH, Münster, Germany) was diluted in water and was administered i.p. to mice at 1.5 mg/kg/day, beginning the day after tumor-cell injection. This rapamycin dose and schedule have been shown to produce steady-state serum levels in a range similar to that used on a long-term basis in organ transplantation to prevent allograft rejection (17). 5-FU (Gry-Pharma, Kirchzarten, Germany) was diluted in saline and administered i.p. at 100 mg/kg on days 3 and 10; in experiments ended on day 10, the second 5-FU injection was given on day 8. When experiments were extended to 20 days, the first two injections were given on days 3 and 10, and a third injection of 5-FU was given at a half-dose (50 mg/kg) on day 17. The 100 mg/kg dose has been reported to be the maximum tolerated dose in mice (24).

Immunohistochemical Ki67 Staining for Tumor Cell Proliferation. Paraffin-embedded tissue sections were prepared and were labeled first with a Ki67-specific monoclonal rat antibody (DAKO A/S, Glostrup, Denmark), followed by staining with a biotinylated antirat immunoglobulin antibody (DAKO A/S). The biotinylated antibody was then detected histochemically using the DAKO StrepABComplex staining kit. The color reaction was visualized with diaminobenzidine, and tissues were counterstained with Mayer's hematoxylin.

In Vitro Cell Viability and Growth Assays. Specific analysis of CT-26 cell viability was performed directly in 6-well culture dishes using a standard ethidium bromide-acridine orange staining method. Briefly, CT-26 cells were cultured for 18 h in the presence or absence of different concentrations of rapamycin and 5-FU. A concentrated (100 \times) ethidium bromide-acridine orange solution was diluted to 1 \times in each well, and fluorescence microscopy was performed. Ten random low-

power fields were examined for each culture well by capturing images of green (viable) and orange-red (nonviable) fluorescence, using two different fluorescence filters. From these images, the percentage of dead cells per field was calculated.

To examine *in vitro* tumor cell growth, CT-26 cells were seeded at 80,000/well in 6-well plates. Rapamycin or 5-FU was then added to the cultures for 48 h, after which, cells were trypsinized and viable cells counted with a hemacytometer (trypan blue exclusion). It is notable that a plating density of 80,000/well was selected because, after 48 h of culture, CT-26 cells do not reach a confluency over 80%, indicating a test period in which active cell proliferation is permitted.

HUVECs and Cell Migration Assay. Human umbilical vein endothelial cell (HUVEC) cultures were purchased from PromoCell (Heidelberg, Germany) and were maintained in Falcon "surface-modified," polystyrene flasks with growth factor supplemented ("Supplement Pack," PromoCell) endothelial-cell basal medium (PromoCell) containing 2% fetal bovine serum, as detailed by the manufacturer.

Migration of HUVECs was assessed using a modified Boyden chamber assay. HUVECs (6×10^4 cells/well) were seeded into the upper well of a chamber system (Becton Dickinson Falcon cell culture insert; BD Biosciences, Heidelberg, Germany) on a fibronectin-coated (Sigma-Aldrich Chemicals, Deisenhofen, Germany) polyethylene terephthalate membrane with 8- μ m pores. Recombinant VEGF₁₆₅ (R&D Systems, Wiesbaden, Germany) or dRib (Sigma-Aldrich Chemicals) was added as a chemo-attractant into the lower well at indicated concentrations. The inhibition of VEGF or dRib-induced chemotaxis was assessed after including rapamycin at relevant doses. Migration through the membrane was determined after 5 h of incubation at 37°C by fixing, staining (H&E), and counting the migrated cells. Each culture condition was performed in triplicate.

Aortic Ring Assay. Aortic ring assays were performed using a modification of the technique reported by Nicosia *et al.* (25). Briefly, thoracic aortae were harvested from 6–8-week-old male ACI rats (Harlan Winkelmann) and sectioned into 1-mm slices, which were placed immediately on Matrigel-coated 24-well plates. HEPES-buffered DMEM containing dRib, rapamycin, or both substances was added and the plate was incubated at 37°C, 5% CO₂, for 4 days. Fresh medium with respective additives was reintroduced into the cultures on day 2. Phase-contrast photomicrographs of the rings were taken to record the formation of vessel sprouts.

Western Blotting. For the TP Western blotting analysis, extracts were prepared from tissue samples or cell cultures with SDS sample buffer. Equal amounts of protein extract were separated on polyacrylamide SDS gels, transferred, and probed with mouse anti-TP antibody (Kamiya Biomedical Company, Seattle, WA). Detection of the primary antibody was done with an antimouse horseradish peroxidase antibody (DAKO A/S) using the ECL Western blotting system (Amersham, Freiburg, Germany). For Western blot analysis of p70/S6 kinase proteins in HUVEC cultures, rabbit antibodies (New England Labs, Frankfurt, Germany) against p70/S6 kinase phosphorylated at the Thr³⁸⁹ site, or against total p70/S6 kinase, were used in the primary reaction. Detection was performed as before with a secondary goat antirabbit horseradish peroxidase antibody (New

Table 1 Tumor and metastasis incidence after orthotopic CT-26 tumor cell injection

Mice received orthotopic injections of CT-26 colon adenocarcinoma cells, and treatment was initiated. Rapamycin was given i.p. at 1.5 mg/kg/day starting the second day, and 5-FU was given twice (days 3 and 10) at 100 mg/kg. Mice were sacrificed on day 14.

Treatment	Orthotopic tumor	Lymph node metastasis	Liver metastasis
Control (saline)	7/7	7/7	4/7
5-FU ^a	8/8	3/8	1/8
Rapamycin	7/8	0/8	0/8
5-FU + rapamycin	1/8	0/8	0/8

^a 5-FU, 5-fluorouracil.

England Labs). β -actin was detected in the same way with a primary goat antibody against β -actin, followed by a donkey antigoat horseradish peroxidase antibody (antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Notably, wortmannin (Sigma-Aldrich Chemicals) was used in some of the HUVEC cultures at 100 nM to block phosphatidylinositol 3-kinase (PI3K) signaling.

Statistical Analysis. Data are given as the mean \pm SE in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student's *t* test was performed.

RESULTS

Effect of Rapamycin and 5-FU on Colon Adenocarcinoma Growth and Metastasis. Experiments were performed to test the hypothesis that rapamycin and 5-FU in combination are effective against colon adenocarcinoma. To test this theory and to simulate cancer cell dissemination prior to, or after, colon surgery BALB/c mice were orthotopically inoculated with syngeneic CT-26 colon adenocarcinoma cells. Development of symptoms from the bowel due to the growth of tumors in control mice necessitated the sacrifice of animals at day 14 after tumor-cell implantation. Necropsies of the mice revealed that all of the control and 5-FU-treated mice developed tumors in the colon (Table 1), whereas one mouse treated with rapamycin alone did not develop a macroscopic tumor. Remarkably, however, <13% of mice treated with rapamycin in combination with 5-FU developed a colonic tumor. We also analyzed the weight of tumors removed from each of the mice in this experiment (Fig. 1). Although tumor growth was inhibited by 59% with 5-FU treatment, rapamycin alone inhibited tumor growth to a greater degree (89%), particularly when used in combination with 5-FU (99%). With regard to metastatic tumor growth, nearly all control animals had lymph node and liver metastases, whereas rapamycin alone (or combined with 5-FU) prevented tumor metastases at both sites (Table 1). 5-FU treatment alone reduced, but did not eliminate, metastasis incidence. An advantage of combined rapamycin and 5-FU treatment on metastasis incidence could not be determined with these initial experiments because rapamycin alone blocked their early appearance.

Effect of Rapamycin and 5-FU on the Growth of Liver Metastases. In our next series of experiments, our goal was to test these same drugs in an experimental situation in which we assumed that some tumor cells do gain access to the portal venous system, simulating the frequent problem of colorectal

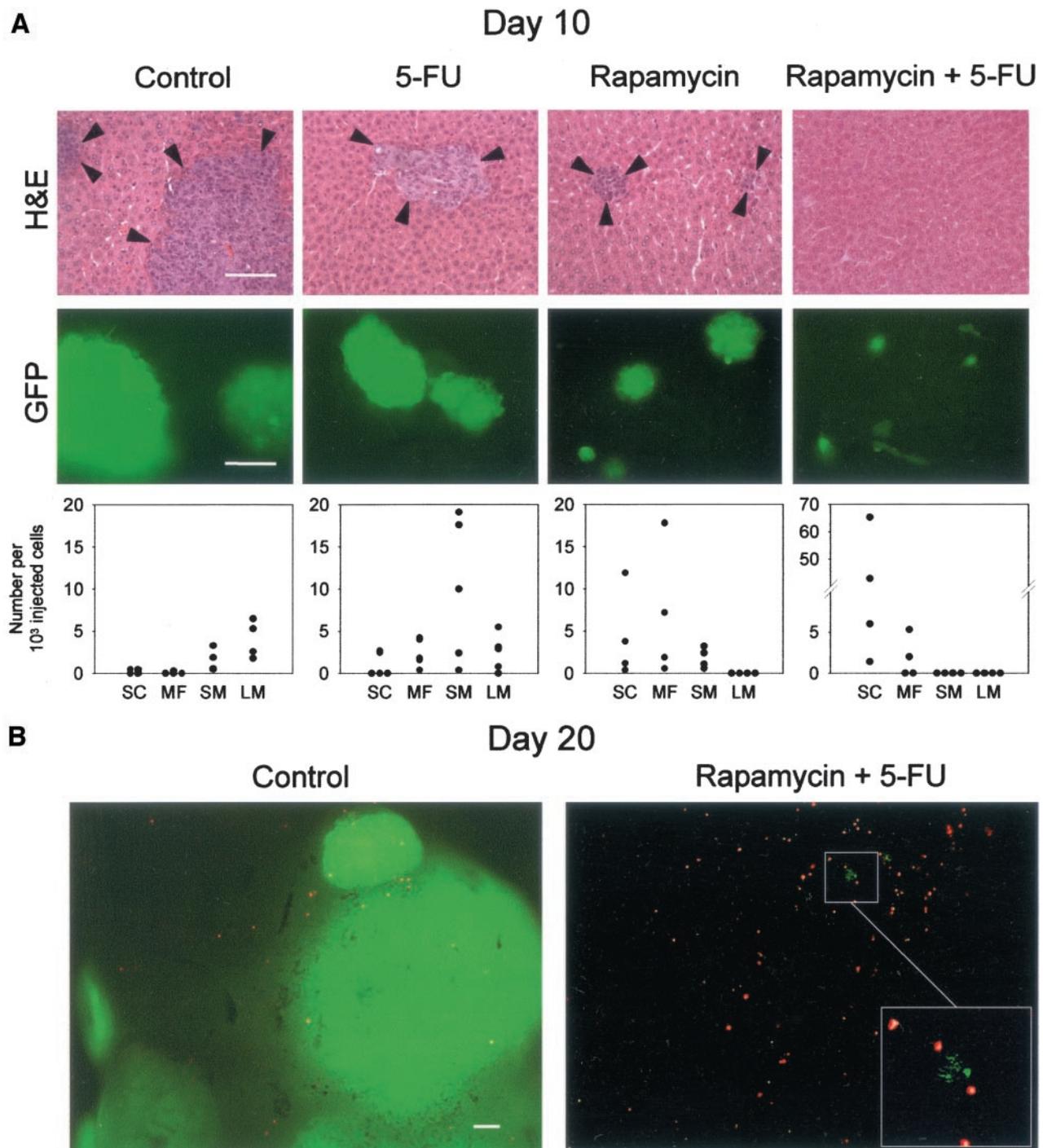


Fig. 2 Effect of rapamycin and 5-fluorouracil (5-FU) on the development of CT-26 liver metastases. Mice were given injections intraportally of green fluorescent protein (GFP)-expressing CT-26 tumor cells, and treatment with rapamycin and 5-FU was initiated. *A, top panels*, standard H&E staining of liver tissue from mice sacrificed on day 10 (*arrowheads*, tumors; *scale bar*, 100 μ m). *Middle panel*, fluorescent microscopic views of the liver surface show the presence of CT-26 tumors with the various treatment regimens. Note that although no tumor cells could be identified by H&E staining with rapamycin + 5-FU treatment, numerous individual GFP-positive tumor cells could be seen by fluorescence. *Bottom panel*, the quantification results of different GFP-expressing CT-26 cell foci in the liver of each of the mice tested (SC, single cells; MF, multicellular foci; SM, small metastases; LM, large metastases). *Each data point*, the number of each type of foci for an individual mouse. *B*, a second group of mice were treated with the rapamycin and 5-FU combination therapy, but animals were sacrificed at 20 days. Low-magnification photos of liver from a control and treated mouse are shown. Bulky, green-fluorescent masses of tumor were present in controls, whereas only a rare green-fluorescent cell, or group of cells, could be seen with rapamycin + 5-FU treatment, as outlined and magnified in the photo insert. A large number of simultaneously injected red-fluorescent marker beads can be seen in the photo using a long band-pass filter, confirming the successful injection of tumor cells. Analysis of hepatic replacement area showed $32.3 \pm 5.1\%$ replacement of liver with tumor in the control group, and only $0.03 \pm 0.01\%$ in rapamycin + 5-FU-treated mice at 20 days. *Scale bar*, 100 μ m.

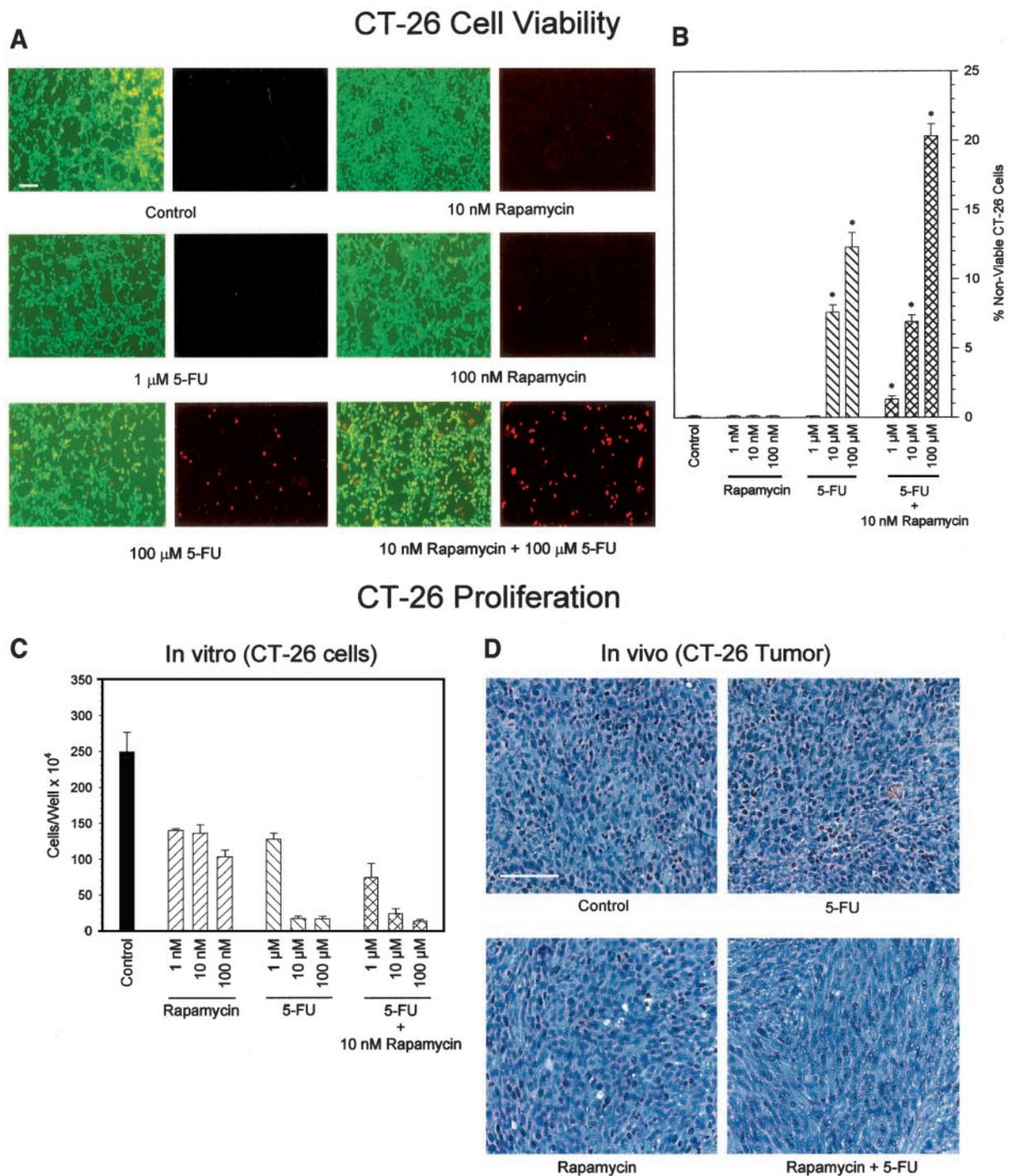


Fig. 3 Effect of rapamycin and 5-fluorouracil (5-FU) on CT-26 viability and proliferation. **A**, CT-26 cell cultures were incubated with various combinations of rapamycin and 5-FU, and cell viability was determined after 18 h with ethidium bromide–acridine orange staining. Representative treatment groups, *left photo*, green fluorescence (viable cells); *right photo*, the orange-red fluorescence (nonviable cells). Scale bar, 100 μ m. Quantitation of these results is shown in **B** for all of the conditions tested (*, $P < 0.001$ versus control). **C**, in a separate experiment, the number of viable cultured CT-26 cells was counted after 48-h exposure to rapamycin or 5-FU. All of the treatment groups showed significantly lower cell numbers versus controls ($P < 0.03$). Ten nM rapamycin was used for combination experiments because it represents a long-term sustainable *in vivo* drug level. **D**, orthotopic CT-26 tumors from the different treatment groups were evaluated for proliferation (day 14) by colorimetric (dark brown color) Ki67 staining. Treatment with rapamycin and rapamycin + 5-FU, but not 5-FU alone, reduced the proliferation rate within these tumors. The mean number of Ki67 positive cells per high-power field was as follows: Control, 48.3 ± 3.9 ; 5-FU, 87.5 ± 7.8 ; Rapamycin, 25.6 ± 2.3 ; Rapamycin + 5-FU, 15.8 ± 4.5 . Scale bar, 100 μ m.

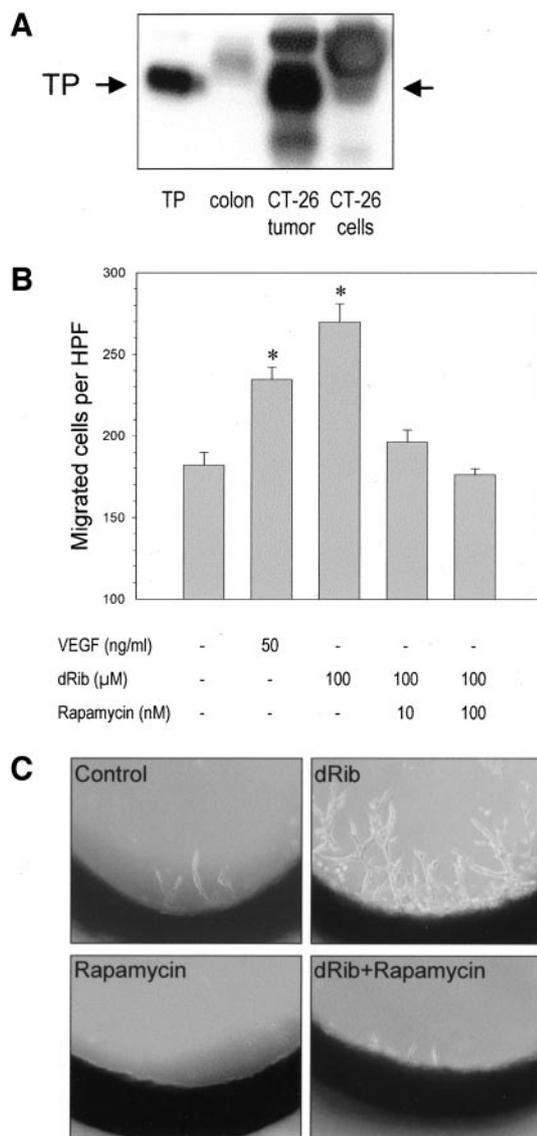


Fig. 4 Expression of thymidine phosphorylase (TP) in CT-26 tumors and the effects of rapamycin on 2-deoxy-D-ribose (dRib)-induced angiogenesis. **A**, a Western blot analysis specific for TP protein was performed. Samples were as follows: purified TP standard (3 ng; R&D Systems), isolated mouse colon epithelial cells as a negative control (colon; Ref. 40), lysate from a CT-26 tumor, and lysate from cultured CT-26 cells. **B**, migration of endothelial cells (human umbilical vein endothelial cells), in the presence of different combinations of vascular endothelial cell growth factor (VEGF), dRib, and rapamycin, was assessed using a modified Boyden chamber assay. *, $P < 0.05$ versus no treatment. high-power field (HPF). **C**, the effect of dRib and rapamycin on rat aortic ring formation was tested and representative photos are shown. Angiogenesis, indicated by the formation of tubular structures from the aortic wall, was induced in the presence of dRib, and dRib stimulation was blocked with rapamycin.

promotes vessel formation through a rapamycin-sensitive pathway. Because dRib has been shown to stimulate endothelial cell migration (13, 14), we tested the migration of HUVECs in Boyden chambers. Our results show that in comparison with VEGF, dRib stimulates HUVEC migration as well, or better.

Importantly, rapamycin addition to cultures at concentrations relevant to *in vivo* dosing (10–100 nM) completely blocked dRib-induced HUVEC migration (Fig. 4B). Similarly, dRib induced endothelial cell sprouting from cultured rat aortic rings, and this activity was abrogated by 10 nM rapamycin (Fig. 4C). Together, these data suggested that stimulation of angiogenesis by dRib is mediated through an mTOR-dependent pathway.

At present, the mechanism by which dRib signals, or stimulates, endothelial cells is not known. However, based on the central role that mTOR-sensitive p70/S6 kinase intracellular signaling plays in endothelial cell proliferation, transformation, and survival, and on our data showing that dRib stimulation of endothelial cells is blocked with rapamycin, we hypothesized that dRib may mediate its angiogenic activity via p70/S6 kinase activation. Using a Western blotting analysis to detect phosphorylation of p70/S6 kinase at the Thr³⁸⁹ site, previously correlated with p70/S6 kinase activation (26), we could show that dRib is capable of activating p70/S6 kinase in a dose-dependent manner, similar to stimulation of HUVECs with VEGF (Fig. 5). Furthermore, mTOR inhibition with rapamycin completely blocked dRib-induced p70/S6 kinase phosphorylation of Thr³⁸⁹. The p70/S6 kinase phosphorylation induced by dRib could also be reversed by blocking the more upstream signaling intermediate PI3K, with wortmannin. These data link dRib stimulation of angiogenesis to activation of the PI3K–mTOR–p70/S6 kinase intracellular signaling pathway.

DISCUSSION

Accumulating evidence in the literature indicates expression of TP by tumors is an important factor influencing the treatment and clinical outcome of various different types of cancer, including breast, pancreatic, and colorectal cancer. An ironic dilemma potentially exists with the treatment of TP-expressing tumors in that pyrimidine analogs, such as 5-FU, are converted to active cytotoxic metabolites by TP, thereby increasing the effectiveness of the drug's anticancer action. However, an opposite tumor-promoting effect of TP is also known, which is associated with the proangiogenic effect of the TP-generated molecule, dRib. Importantly, results from the present study indicate that the proangiogenic mechanism of dRib can be

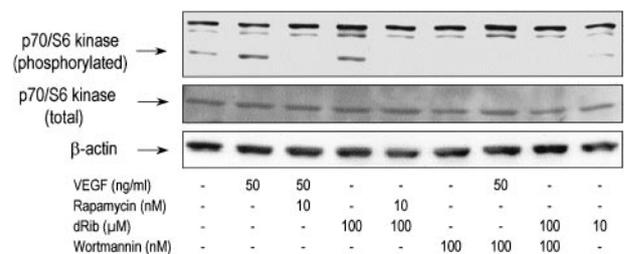


Fig. 5 Effect of 2-deoxy-D-ribose (dRib) on activation–phosphorylation of p70/S6 kinase. A Western blotting analysis was performed on human umbilical vein endothelial cells cultured in the presence of various combinations of vascular endothelial cell growth factor (VEGF), rapamycin, dRib, and wortmannin. Cell extracts were separated on polyacrylamide-SDS gels and were transferred and probed with antibodies against phospho-specific p70/S6 kinase (Thr³⁸⁹), total p70/S6 kinase, or β-actin.

disabled with the mTOR-inhibitor rapamycin, presumably without interfering with the ability of TP to convert pyrimidine analogs into their most active cytotoxic forms. Therefore, the use of rapamycin blocks dRib-induced angiogenesis, removing a potential escape mechanism available for TP-expressing tumors under pyrimidine analog chemotherapy.

The potency of a combined rapamycin–5-FU treatment strategy was demonstrated in our results by the dramatic reduction in colon adenocarcinoma tumor growth in mice. Moreover, rapamycin use as a single agent in these studies was more effective at reducing tumor growth than was 5-FU alone, suggesting that TP-expressing colon tumors are highly sensitive to agents targeting angiogenesis. Although we cannot exclude non-angiogenesis-related effects on the tumor by rapamycin, including reduced tumor cell growth *in vitro*, our data do show that rapamycin is not directly cytotoxic against CT-26 adenocarcinoma cells *in vitro*. Therefore, considering our recent findings that angiogenesis in different tumors is markedly inhibited by rapamycin (17), our *in vivo* observations are consistent with rapamycin acting at least partially via antiangiogenic effects. Interestingly, however, analysis of tumors taken from rapamycin-treated mice in the present study demonstrated lower levels of proliferation by Ki67 staining than in controls or 5-FU-treated mice, suggesting an indirect antiproliferative effect of rapamycin. Presently, our data cannot explain this result, but at least one possible explanation is that the antiangiogenic effect deprives the surviving tumor cells of oxygen and nutrients, thus limiting cell proliferation. In fact, studies indicate that the proliferation rate of cancer cells decreases proportionally to their distance from the nearest capillary bed (27, 28). Therefore, antiangiogenic substances not only have the potential to directly block development of vessels in tumors, they may also indirectly prevent cell division for tumor expansion.

Indeed, data from our study suggest that angiogenesis plays a central role in at least one possible mechanistic explanation for why the combination of rapamycin and 5-FU is effective against colon adenocarcinoma. A key to the explanation is that our results are the first to show dRib uses the mTOR–p70/S6 kinase intracellular signaling pathway in endothelial cells. Therefore, we hypothesize a novel putative mechanism whereby TP-generated dRib (12), which promotes tumor growth by angiogenesis (13, 29), is neutralized by rapamycin's specific inhibition of its signaling to endothelial cells, and, at the same time, TP maintains its ability to convert 5-FU into derivatives more active against cancer. Although the mechanism by which dRib activates the mTOR–p70/S6 kinase intracellular signaling pathway is presently unknown, a potential explanation could be related to a recent report showing that p70/S6 kinase can be phosphorylated via a hydrogen peroxide–PI3K–mTOR signaling pathway, after UV radiation (30). This report is particularly relevant to our study because of clear evidence from other studies that dRib leads to the generation of reactive oxygen species, including hydrogen peroxide (31). In this same respect, TP-transfected cells have been shown to induce hemoxygenase-1, which is known to be sensitive to oxygen stress mediated by reducing sugars (15), such as dRib. Therefore, pending more in-depth studies, the missing link between dRib and the PI3K–p70/S6 kinase pathway could be related to the generation of reactive oxygen species by dRib.

Although data from the present study show that rapamycin inhibits dRib-induced angiogenesis and that TP was expressed in CT-26 tumors, a TP-dependent effect can only be indirectly implied at this stage. Unfortunately, confirmation that at least part of rapamycin's action is due to TP presents a difficult experimental problem because the enzyme is produced not only by cancer cells but also by normal stromal cells (8) and, furthermore, is known to be locally produced in high amounts near the invading edge of tumors (32). Therefore, for instance, the use of TP-transfected tumor cell lines for *in vivo* studies would not provide definitive answers because of the local-regional contribution of TP-expressing stromal tissue. In fact, this was likely the situation with CT-26 cells in our experiments, in which relatively low TP expression was observed on tumor cells *in vitro*, compared with high expression levels in whole orthotopic tumors (Fig. 4A). Therefore, to better understand the degree to which TP and dRib are involved in rapamycin's antiangiogenic effect on cancer, direct chemical TP inhibition or the development of small interfering RNAs could prove to be useful. However, it must be added that direct inhibition of TP further complicates the study of combination therapy because of the predicted interference with both 5-FU activity and angiogenesis. Certainly, each aspect of this complex physiological scenario will require further investigation.

Other mechanistic considerations become apparent when analyzing the promising therapeutic potential of combined rapamycin and 5-FU on the portal venous spread of colon-derived tumor cells. New treatment strategies that contain or destroy liver metastases from colon cancer will be vital to reduce the high mortality currently associated with this disease. Results from our experimental simulation of colon cancer liver metastasis were consistent with the hypothesis that rapamycin inhibits angiogenesis. These conclusions are based on the observation that rapamycin treatment alone generally held tumor cell growth to multicellular foci of cells below the size generally required for angiogenesis (33). However, the tracking of individual GFP-expressing tumor cells suggests a different effect of 5-FU. Areas of tumor cell growth within the liver of 5-FU-treated mice were reduced in size compared with the growth in controls, but a substantial number of areas were of a size requiring angiogenesis and were consistently larger than those observed with rapamycin treatment. These results, combined with our *in vitro* data showing a dose-dependent cytotoxic effect on tumor cells by 5-FU, suggest the tumor reduction by 5-FU is due to direct effects on the tumor cells and not from an antiangiogenic effect. Furthermore, the effect of 5-FU on HUVEC proliferation is weak in comparison with the effect of rapamycin (data not shown). Notwithstanding these arguments, an intriguing growth pattern of tumor cells found in the livers of mice treated with both rapamycin and 5-FU raises other important mechanistic issues. In rapamycin + 5-FU-treated mice, nearly all tumor cells were existing as single cells after day 10, and later disappeared (20 days) or showed no signs of expansion to metastases. Other studies using a similar metastasis assay to the liver show that tumor cells continue to exist in a dormant state (23, 34) for at least 3 weeks, although some of these cells during the same time period grow into macroscopic metastases. Therefore, our study suggests that the use of rapamycin in combination with 5-FU may substantially reduce the number of solitary, potentially

dormant, tumor cells and, importantly, may prevent the development of the cells into metastases. How this occurs is presently unknown; however, one possibility is that rapamycin, which can be administered safely on a daily basis at effective doses, could inhibit rapid overexpansion of tumors by its antiangiogenic and indirect antiproliferative effect. The role of repeated 5-FU administration could then be to actively destroy growing tumor nests and cells that do emerge from dormancy. Because angiogenesis plays a central role in the growth of deadly colon cancer-derived liver metastases (27) and because increased VEGF and TP expression are closely correlated with this complication, the use of rapamycin in combination with 5-FU should be considered in these patients.

In a more broad sense, this basic therapeutic strategy could also be applied to other forms of cancer that both respond to pyrimidine analogs and express relatively high levels of TP. Indeed, of the various angiogenic factors, including VEGF and basic fibroblast growth factor, TP expression has correlated consistently with vascular density and tumor progression in both breast and pancreatic cancer (8, 12). Moreover, newer generation pyrimidine analogs such as capecitabine (24, 35) have been rationally designed to generate cytotoxic 5-FU metabolites preferentially in the tumors, based on the high expression of TP within a tumor (36). Perhaps combination therapy with rapamycin in this situation could be a critical factor to restrain TP–dRib-mediated angiogenesis, thus maximizing the chance for longer-term survival or an otherwise nonobtainable cure. Another consideration is that other cytotoxic drugs such as the taxanes and cyclophosphamide are known to up-regulate TP expression in tumors (37). Therefore, rapamycin use opens up the possibility that these TP up-regulators could be used in combination with pyrimidine analogs to boost cytotoxicity within the tumor, without risking increased tumor growth from the proangiogenic effect of TP-generated dRib. Finally, it is important to consider the practical feasibility of rapamycin use for cancer treatment. The present study and our previous work (17) clearly show that rapamycin has an optimal antiangiogenic effect at normal immunosuppressive doses used in mice. In humans, rapamycin can be administered to organ transplant patients at similar daily doses for several years with relatively mild side effects (18). Our study using rapamycin may be particularly relevant in light of new clinical data indicating that combination therapy for colorectal cancer metastases using an anti-VEGF antibody (bevacizumab) is an effective treatment option (16, 38). Interestingly, we have previously shown that rapamycin not only inhibits VEGF production but it blocks VEGF stimulation of endothelial cells (17); moreover, the present study adds that dRib stimulation of angiogenesis is abrogated by rapamycin, potentially through the inhibition of the common PI3K–p70S6 kinase pathway, also used by VEGF (39). Therefore, with the understanding that rapamycin treatment in humans is practical, and considering the mechanistic logic for combining its specific antiangiogenic activities with pyrimidine derivatives, this drug regimen should be considered for clinical use to treat colon adenocarcinomas and other types of TP-expressing cancers.

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Blockage of 2-Deoxy-d-Ribose-Induced Angiogenesis with Rapamycin Counteracts a Thymidine Phosphorylase-Based Escape Mechanism Available for Colon Cancer under 5-Fluorouracil Therapy

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