

Rifampicin as an Oral Angiogenesis Inhibitor Targeting Hepatic Cancers

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Abstract

Angiogenesis is an important therapeutic target in cancer, and to fully exploit its therapeutic potential, combination chemotherapeutic/antiangiogenic regimens should be optimized and delivered earlier to more patients. Ideally, this could be done by a single potent oral agent with established safety. Rifampicin, a semisynthetic antibiotic derived from the rifamycins, is one of the most commonly used pharmaceutical compounds worldwide in the treatment of tuberculosis. Here, we present the effects of oral rifampicin on human cancer progression and its antiangiogenic properties, which were comparable to the angiogenesis inhibitor endostatin. Clinically, low-dose p.o. administration of rifampicin to six high-risk patients with hepatitis C virus–related liver cirrhosis resulted in a single occurrence of hepatocellular carcinoma during the follow-up period of 97.3 ± 29.1 (mean \pm SD) months. Experimentally, rifampicin rapidly and markedly down-regulated the expression of a wide spectrum of angiogenesis-associated genes in growing human microvascular endothelial cells, thereby suppressing endothelial cell proliferation and migration. Rifampicin, at higher concentrations, also directly inhibited the growth of a variety of human cancer cells. P.o. administration of rifampicin significantly inhibited *in vivo* growth and metastases of subcutaneous human cancer xenografts. Thus, the potent antiangiogenic properties of oral rifampicin therapy were effective in suppressing cancer progression. It provides a promising new addition to antiangiogenic strategies for designing human cancer therapies. Considering the clinical pharmacokinetics of rifampicin, which enters the enterohepatic circulation and undergoes subsequent hepatic accumulation, it may be especially beneficial as an antitumor agent targeting hepatobiliary tumors. [Cancer Res 2009;69(11):4760–8]

Introduction

Cancer progression and metastasis depend on recruitment of new capillaries from preexisting vessels, a process known as angiogenesis (1). Because tumors that are unable to elicit angiogenesis remain in a dormant state and fail to grow beyond a few millimeters in size (2), the multistep process of angiogenesis has fostered an intense search for agents that might inhibit this process (3). Although tumor growth can be stunted by a variety of single angiogenesis inhibitors (2),

combination antiangiogenesis protocols can be more effective than single-agent therapies (3, 4). Furthermore, the combination of traditional chemotherapy with antiangiogenesis agents, which targets the endothelial cells of the growing tumor vasculature, as well as the growing tumor cells themselves, has been shown to minimize the resistance and adverse effects of chemotherapeutic agents (4). However, successful clinical application of angiogenesis inhibitors, irrespective of monotherapy or combination therapy, requires long-term administration, but most of these entering clinical trials are costly, large molecular weight proteins requiring parenteral administration (3, 5).

Rifampicin is a semisynthetic antibiotic derived from the rifamycins, the common structure of which is a naphthohydroquinone or naphthoquinone chromophore spanned by an aliphatic chain. Rifampicin binds to and activates pregnane X receptor, which affects cytochrome *P*450, glucuronosyltransferases, and *p*-glycoprotein activities (6). It is extensively used to treat tuberculosis and for controlling pruritus in patients with chronic cholestatic liver disease (7). Our encounter with a patient with pulmonary tuberculosis combined with multiple hepatocellular carcinomas (HCC), who seemed to show blunted tumor progression while receiving rifampicin, prompted us to study whether oral rifampicin had a significant tumor-suppressive effect. Our data revealed involvement of both antiangiogenic properties and a direct tumor cell inhibitory effect of rifampicin in suppressing human cancer progression, and, considering its clinical pharmacokinetics, oral rifampicin may be especially beneficial in targeting hepatobiliary tumors. Furthermore, we found that rifampicin exerts its antiangiogenic effect through inhibition of the expression of angiogenesis-associated genes in a manner comparable to the action of endostatin, an endogenous angiogenesis inhibitor known to down-regulate a variety of growth- and angiogenesis-related genes in a wide range of endothelial lineage cells (8, 9).

Materials and Methods

Clinical follow-up. Six patients were followed up monthly at Tokyo Medical and Dental University Hospital, after a diagnosis of liver cirrhosis by either liver biopsy and/or a combination of clinical features. During monthly visits, they underwent physical examination, urinalysis, and complete blood count, with measurement of serum α -fetoprotein, prothrombin time, activated partial thromboplastin time, and serum chemistry. All patients received a liver ultrasound, magnetic resonance imaging (MRI), and/or computed tomography (CT) at least every 6 mo. The protocol was approved by the Ethical Committee of Tokyo Medical and Dental University.

***In vivo* human cancer xenograft experiments.** CW-2 human colon cancer xenograft experiments were conducted in accordance with the Guidelines for Animal Experimentation, and approved by the Animal Care and Use Committee of Tokyo Medical and Dental University. Male athymic BALB/c Slc-nu mice (Japan SLC) were given free access to sterile rodent chow and water. CW-2 cells (1.2×10^6 per animal) were implanted s.c. in the hind flank region in 8-wk-old mice. When tumors

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Table 1. Characteristics of patients receiving long-term, low-dose oral rifampicin

Patient no.	Sex	Age (y)	Platelet ($\times 10^4/\text{mm}^3$)	Serum albumin (mg/dL)	Serum ALT (IU/L; normal 8–48)	α -Fetoprotein (ng/mL; normal 0–10)	HCV		Associated diseases	Total follow-up period (mo)	Rifampicin therapy period (mo)
							Genotype	Copy no. (kEq)			
At the start of rifampicin											
1	Male	56	5.9	3.2	82	64.0	Ib	1,500	MR due to ruptured chordae tendineae, hypertension	209	90
2	Female	66	16.5	3.8	149	25.2	Ib	20,000		161	82
3	Female	64	13.4	3.8	139	93.3	Ib	1,600	Type 2 diabetes	121	103
4	Female	69	5.3	3.3	149	11.1	Ib	620	Hypertension	149	62
5	Female	60	8.6	4.0	129	3.3	Ib	5,500		187	149
6	Female	57	6.1	3.7	120	14.7	Ib (mutant)	110	Graves diabetes	135	98

Abbreviations: ALT, alanine aminotransferase; MR, mitral regurgitation.

had reached 200 mm³, animals were moved to a cage supplied with sterile drinking water containing either 0.2 mg/mL rifampicin/0.25% DMSO or 0.25% DMSO alone. Tumor growth during the treatment period was monitored by measuring the tumor mass on the animals using vernier calipers thrice a week. Tumor volume was calculated as the product of length \times (width)² \times 0.52. The well-established Lewis lung carcinoma xenograft experiment was done by Shibayagi, Inc. LL/2 cells (10^6 per animal) were s.c. injected into the flank of 8-wk-old male athymic BALB/c AnNCrj-*nu/nu* mice (Japan Charles River). Animals were started on 0.8 mg rifampicin, 3-formyl rifamycin SV, rifamycin SV, or double-distilled water (ddH₂O) via a gastric tube once daily during weekdays when the tumor volume had reached 200 mm³. The tumor volume was calculated thrice a week as described above. Each group contained seven mice. On termination of the efficacy portion of the experiment, animals were euthanized; liver and lungs were excised, sectioned, and photographed; and the ratio of metastatic to total section area was calculated. Tumor volume and metastasis data of treated groups were compared with those of the control group using Wilcoxon's rank sum test.

In vivo transsplenic liver metastasis model. The growth of the A549 human lung cancer cells metastasized into liver was evaluated by Panapharm Laboratories, essentially as described previously (10). In brief, 1.5×10^6 cells in 0.05 mL PBS were injected into the medial splenic tip of the anesthetized BALB/cA Jcl-*nu/nu* mice (Japan Crea Laboratory) through a left lateral flank incision. No significant bleeding or extravasation was encountered. After 10 min, the spleen was removed to avoid intrasplenic tumor growth. Four days after injection, the treated mice were divided into four groups and were started on 0, 50, or 100 mg/kg/d rifampicin or 30 mg/kg/d 5-fluorouracil (5-FU) via a gastric tube once daily during weekdays. Rifampicin was dissolved to 0, 5, or 10 mg/mL using 0.5% carboxymethylcellulose sodium solution, and 10 mL/kg/d was administered at 10 a.m. after a 2-h fast. After 3 wk, the animals were anesthetized; blood was withdrawn for the measurement of CA15-3; and the animals were sacrificed to examine macroscopic metastasis as well as micrometastasis after fixation.

Mouse dorsal air sac assay. Malignant tumor cell–induced angiogenesis was assessed by Panapharm Laboratories (Kumamoto, Japan) as described (11). A Millipore chamber prepared by covering both sides with Millipore filters (0.45-mm pore size) was filled with 5×10^6 Sarcoma 180 cells suspended in 0.15 mL PBS. The tumor cell–containing chamber was

implanted into a subcutaneous dorsal air sac formed in a 9- or 10-wk-old female Crlj:CD1 (ICR) mouse by injecting ~ 8 mL of air. Each group of mice treated with tumor cells was given via a gastric tube with 0, 100, or 200 mg/kg/d rifampicin once a day or with 200 mg/kg/d silibinin twice daily as a positive control for 5 d. Rifampicin was dissolved to 10 or 20 mg/mL using 0.5% carboxymethylcellulose sodium and silibinin to 10 mg/mL (0.9% NaCl, 3% ethanol, 1% Tween 80, and 6.6 mmol/L NaOH). The nontumor control group, which received chambers containing PBS in place of Sarcoma 180 cells, was given the vehicle alone. On day 5, the implanted chambers were removed from the subcutaneous fascia of the treated animals, and then a black ring (Sanko Kagaku, Hiroshima) with the same inner diameter as the Millipore ring was inserted at the same site. The angiogenic response was assessed under a dissecting microscope by counting newly formed blood vessels >3 mm in length within the area encircled by the black ring. The neovessels were morphologically distinguishable from the preexisting background vessels by their tortuous nature.

Cell culture. Rat pulmonary arterial endothelial cells (rPEC) and rat aortic endothelial cells (rAEC) prepared from the pulmonary artery and aorta of male Wistar rats, respectively, and diploid rat fibroblasts have been described previously (9, 12, 13). Human dermal microvascular endothelial cells (hMVEC) and human umbilical vein endothelial cells (hUVEC) were purchased from Kurabo and Sanko-Junyaku, and human retinal endothelial cells (hREC) from Cell Systems Corporation. Human colon carcinoma cells, CW-2, human gastric cancer cells, MKN-1, and human liver cancer cells, HepG2, were purchased from RIKEN BioResource Center Cell Bank.

Endothelial cell migration, proliferation, and apoptosis assays. Anchorage-dependent cell migration was assessed using the monolayer-denuding protocol as described previously (14). Confluent cultures of endothelial cells in collagen-coated dishes were pretreated with or without rifampicin for 16 h, and then denuded with a surgical blade. Cells were further incubated in 20% fetal bovine serum (FBS) supplemented with growth factors, whereas phase-contrast photomicroscopy was done at the indicated times at four distinct sites, and the cell migration rate was measured. DNA synthesis in confluent cultures was negligible during the monolayer wounding experiments, and repopulation within 24 h was almost entirely to the result of cell migration (9). Exponentially proliferating cells plated in 24-well plates were incubated for 24, 48, or 72 h in the presence or absence of indicated concentrations of rifampicin, rifamycin SV, or 3-formyl rifamycin SV. Cells were then released from culture plates by trypsinization, and cell numbers counted using a Sysmex CDA-500

Autoanalyzer (Toa Medical Electronics) as described previously (9, 15, 16). Proapoptotic effects were evaluated by adding rifampicin to growing cell cultures and determining caspase-3 levels as described previously (17).

Evaluation of antitumor activity using a human cancer cell line panel coupled with a drug sensitivity database. A human cancer cell line panel, composed of 39 cell lines combined with a database for evaluating the cell growth inhibition profile, originally established according to the method of the National Cancer Institute (18, 19), was done by Takao Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research), as previously described (20, 21). Antitumor activity was assessed by changes in total cellular protein after 48-h rifampicin treatment using a sulforhodamine B assay (20, 22).

Gene inhibition profile determined by real-time quantitative reverse transcription-PCR. Exponentially growing hMVECs or hUVECs supplemented with FBS and growth factors in 6-cm dishes (10^6 cells) were treated with the indicated concentrations of rifampicin, rifamycin SV, and 3-formyl rifamycin SV for the indicated time. Total RNA was extracted using QIAzol (Qiagen), and 1 μ g of RNA was reverse transcribed with the first-strand cDNA synthesis kit (Qiagen). The expression of angiogenesis-associated genes was quantified by a LightCycler (Roche Molecular Biochemicals)-based, real-time, quantitative reverse transcription-PCR (RT-PCR) protocol using Syber Green as described (9, 23). Quantification of *c-myc*, *integrin- α_v* , *ID1*, vascular endothelial growth factor (VEGF), and hepatocyte growth factor was done by TaqMan fluorescence methods using a Universal Probe Library (Roche Molecular Biochemicals) and Chromo 4 Four-Color Real-Time Detection System (Bio-Rad Laboratories). The fluorescence data were quantitatively analyzed by using serial dilution of control samples included in each reaction to produce a standard curve. We have also quantified glyceraldehyde-3-phosphate dehydrogenase and β -actin mRNA levels to confirm technical validity.

Results

Clinical benefit of low-dose, long-term oral rifampicin in six patients with hepatitis C virus-related liver cirrhosis. Since 1994, six patients (one male and five females, 58.2 ± 3.8 years at initial visit), confirmed to have HCV-related liver cirrhosis, volunteered to receive low-dose oral rifampicin therapy (Table 1). They were at high risk for the development of HCC (24); therefore, they received monthly follow-up by a hepatologist (Y.T.) and 4- to 6-monthly examinations using liver ultrasound, MRI, and/or CT. All six patients had HCV genotype 1b with high HCV RNA levels (110–20,000 kIU/mL), suggesting resistance to IFN- α therapy (25).

Patient 1 received IFN- α treatment in 1991 and 1992 without a virological or sustained biochemical response. He had mitral regurgitation as a result of ruptured chordae tendineae but did not receive cardiac surgery because of liver cirrhosis. He developed HCC in the S₄ portion in May 1993 and another in the S₈ portion in November 1993, both of which were treated with transarterial embolization with associated chemotherapy. His serum α -fetoprotein, a commonly used tumor marker for HCC, started to increase, reaching 66 ng/mL in May 1994. He started treatment with rifampicin 300 mg daily, and within 4 months, his α -fetoprotein was reduced to 15 ng/mL. Because this decrease of α -fetoprotein did not necessarily indicate that rifampicin caused regression of undetectably small HCC, his physician (Y.T.) hesitated to let him continue with the antibiotic. They agreed to discontinue rifampicin in September 1995 when liver ultrasonography did not detect any abnormal mass. Three months later, however, HCC of ~ 2.5 -cm

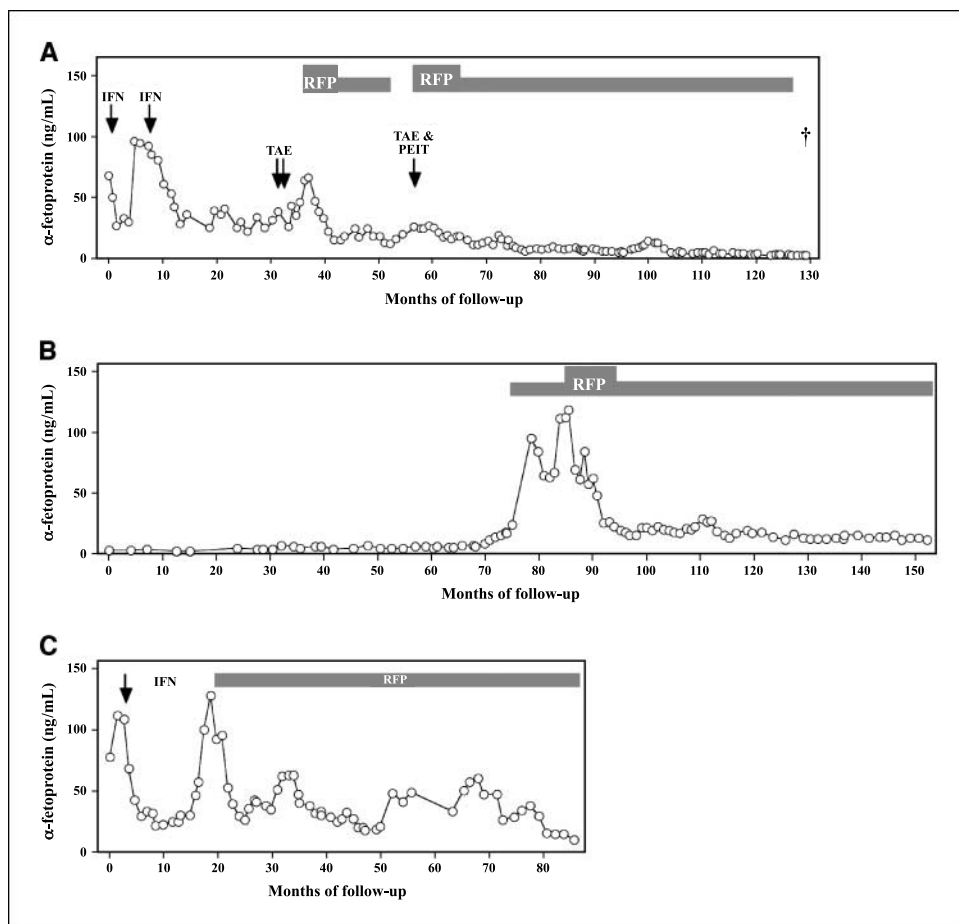
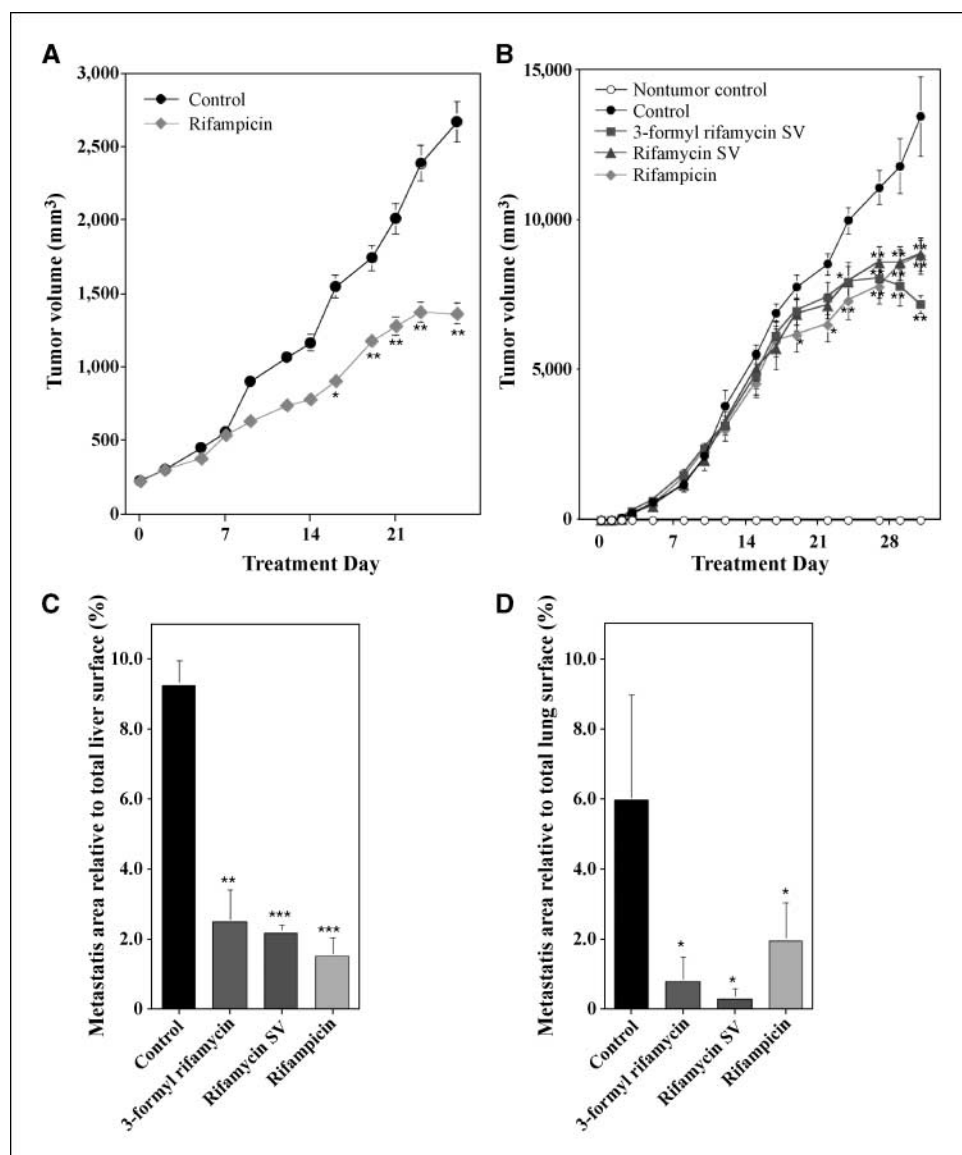


Figure 1. Serial changes of serum α -fetoprotein levels, HCC episodes, IFN- α therapy, and duration of low-dose oral rifampicin therapy in three patients with HCV-related cirrhosis who showed elevated serum α -fetoprotein levels. Patient 1 (A), patient 2 (B), and patient 3 (C) received rifampicin, 150 or 300 mg daily, during the period indicated RFP, and serum α -fetoprotein levels were determined monthly. IFN, conventional IFN therapy; TAE, an episode of HCC treated by thromboarterial embolization; TAE & PEIT, an episode of HCC treated by thromboarterial embolization and percutaneous ethanol infusion therapy.

Figure 2. Inhibition of human cancer progression by p.o. administration of rifampicin. **A**, male immunodeficient mice were implanted with CW-2 human colon cells and oral rifampicin was begun when tumors reached $\sim 200 \text{ mm}^3$ (◆). The tumor volumes were compared with control mice not treated with rifampicin (●). *Points*, mean for seven mice; *bars*, SE. **B**, male immunodeficient mice were implanted with LL/2 Lewis lung carcinoma cells, and oral rifampicin (◆), 3-formyl rifamycin SV (■), rifamycin SV (▲; 40 mg/kg/d), or water (●) was administered via a gastric tube when tumors reached $\sim 200 \text{ mm}^3$. *Points*, mean for seven mice; *bars*, SE. **C** and **D**, at the end of LL/2 xenograft tumor studies, mice were sacrificed, liver and lungs were excised and sectioned, and the ratio of metastatic to total section area was calculated. *Columns*, mean for eight mice; *bars*, SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



diameter developed in the S_8 portion, which was treated with transarterial embolization and percutaneous ethanol injection. Since then, he had just had rifampicin therapy, initially 300 mg and, after September 1996, 150 mg daily. A mass of 1.5-cm diameter was detected on the liver surface S_4 area by MRI in July 1999, for which he received open liver biopsy followed by local microwave ablation in October 1999. Resected liver specimens were histologically negative for HCC. Despite the very high expected recurrence rate (26) and frequent episodes of hepatic encephalopathy, he was totally free from HCC until he died suddenly in January 2002 (Fig. 1A). Patient 2 has been followed since July 1992. Her α -fetoprotein level was normal until May 1998 when it started to increase sharply. She was prescribed oral rifampicin, 150 mg daily, in October 1998, but her α -fetoprotein continued to increase, reaching a peak value of 113 ng/mL in August 1999. This increase led her physician (Y.T.) to increase the rifampicin dose to 300 mg daily, which reduced her α -fetoprotein level to 26.4 ng/mL in March 2000. Her α -fetoprotein never showed a substantial increase thereafter, even after rifampicin was decreased to 150 mg daily

(Fig. 1B). Patient 3 was referred to Y.T. in February 1998. IFN- α therapy reduced her α -fetoprotein but was discontinued because of adverse reactions. In August 1999, her α -fetoprotein increased to 129.2 ng/mL and she was started on rifampicin 150 mg daily, which reduced her α -fetoprotein level thereafter (Fig. 1C). Patients 4 to 6 started oral rifampicin 150 mg daily in the slim hope that it might retard the development of HCC. Patients 5 and 6 received IFN- α before commencing rifampicin therapy, but soon discontinued because of adverse effects. In June 2006, 10 years after the start of rifampicin, patient 5 showed clear-cut evidence of HCCs in the S_1 and S_8 portions, which were treated with transarterial embolization followed by percutaneous radiofrequency catheter ablation. Surprisingly, as of March 2008, this is the only HCC that developed in the six patients during the entire rifampicin therapy period of 97.3 ± 29.1 (mean \pm SD) months. Rifampicin induced neither an adverse reaction nor HCV RNA negativity.

Inhibition of *in vivo* cancer progression by oral rifampicin.

To explore the reasons for such an unexpectedly low occurrence of HCC, which followed long-term, oral rifampicin therapy in the

high-risk liver cirrhosis patients, we first studied the effect of oral rifampicin on cancer progression in well-established mouse xenograft models implanted s.c. with human colon cancer (CW-2) cells. BALB/c Slc-nu mice receiving rifampicin in their drinking water ingested 0.68 ± 0.01 mg rifampicin/d (34.0 ± 0.9 mg/kg/d) and showed a significantly reduced rate of tumor growth compared with those receiving control 0.25% DMSO/ddH₂O (Fig. 2A). Mice receiving rifampicin for 4 weeks showed a mean tumor growth volume 48% less than the control group ($P < 0.05$). We next implanted Lewis lung carcinoma (LL/2) cells subcutaneously in BALB/c AnNCrj-nu/nu mice (27). The mice that were orally given either rifampicin, rifamycin SV, or 3-formyl rifamycin SV via a gastric tube (40 mg/kg/d) showed a significantly slower tumor growth rate ($P < 0.05 \sim 0.01$; Fig. 2B) and reduced metastatic areas in the liver and lung in comparison with those receiving control 0.25% DMSO/ddH₂O ($P < 0.05 \sim 0.01$; Fig. 2C and D). We next injected a human non-small cell lung cancer cell line (A549) into the spleen of BALB/cA Jcl-nu nu/nu mice and, 4 days after inoculation, started giving rifampicin via a gastric tube for 3 weeks. Oral rifampicin reduced the degree of metastasis in hepatic sections as observed photomicroscopically (data not shown) and dose-dependently inhibited the increase in serum CA15-3 concentration, a specific tumor marker produced by metastasized A549 cells (Fig. 3), indicating reduced growth of the hepatic metastatic tumor. The effect of oral rifampicin was comparable to or exceeded that of 30 mg/kg/d 5-FU. These results showed that oral rifampicin potently suppresses the growth of both inoculated and metastasized cancer independently of mouse strain and tumor origin, and that two rifamycins also have a comparable suppressive effect on tumor progression.

In vivo antiangiogenic effect of oral rifampicin. Sarcoma 180 cells induced a robust angiogenic response in the otherwise poorly vascularized subcutaneous area when inserted in a Millipore diffusion chamber and implanted in a dorsal air sac of Crlj:CD1 mice (28). Rifampicin (100 or 200 mg/kg/d) was given p.o. via a gastric tube once daily for 5 days starting after implantation of the Millipore chamber and was compared with those receiving silibinin (200 mg/kg/d), a potent angiogenesis inhibitor (29). Control animals were implanted with a Millipore chamber with or without Sarcoma 180 cells and received ddH₂O. At the end of the treatment, animals were sacrificed, the chambers removed, and vascularization in the skin in contact with the chamber was observed microscopically. Oral rifampicin dose-dependently inhibited newly formed blood microvessels without inducing any toxic effects (Fig. 4). Administration of 200 mg/kg/d oral rifampicin resulted in a 63.5% decrease in the number of neovessels ($P < 0.0001$) and exceeded the effect of silibinin. To highlight the involvement of angiogenesis in *in vivo* rifampicin-induced tumor suppression, its direct antitumor effect was also assessed. CDF1 mice were inoculated i.p. with 10^6 lymphocytic lymphoma cells (P388), and 100, 200, or 400 mg/kg/d rifampicin was administered i.p. on days 1 and 5. Rifampicin did not reduce the median survival of P388-inoculated mice, suggesting no antitumor effect in a model where angiogenesis is not involved. Taken together, the direct antitumor effect of rifampicin can hardly explain the significant inhibition of xenograft tumor progression in immunodeficient mice, but its antiangiogenic effect can reasonably account for this inhibition.

Effects of rifampicin on cell proliferation, migration, and apoptosis. To determine whether rifampicin and rifamycins induce *in vitro* antiangiogenic activities, we tested the antiproliferative activity of rifampicin and rifamycins in vascular endothelial

cells. Rifampicin suppressed FBS-stimulated proliferation of exponentially growing hMVECs (Supplementary Fig. S1A) and hRECs (Supplementary Fig. S1B). The effects were concentration and time dependent and also observed to a lesser degree in rPECs (Supplementary Fig. S1C). 3-Formyl rifamycin SV also exerted an antiproliferative effect on hMVECs, hRECs, and rPECs (Supplementary Fig. S1D–F). However, rifamycin SV showed significant cytotoxic effects on the three types of endothelial cells (Supplementary Fig. S1G–I). The inhibitory effects of rifampicin on VEGF-induced proliferation in quiescent hUEVCs were also concentration dependent (25–100 μ g/mL), but not as potent as in growing endothelial cells ($IC_{50} \sim 60$ μ g/mL).

We determined the antiproliferative effect of rifampicin on human cancer-derived cells and compared their effects with endothelial cells. Rifampicin suppressed the proliferation of CW-2 and HepG2, but the effects were less potent than on endothelial cells (Supplementary Fig. S2). To evaluate the antiproliferative activity of rifampicin on human cancer-derived cells, a panel of 39 human cancer cell lines combined with a database was used (20). This panel was made up of five breast cancer, six glioma, five colorectal cancer, seven lung cancer, one melanoma, five ovarian cancer, two renal cancer, six gastric cancer, and prostate cancer cell lines. Rifampicin at 10^{-4} mol/L (82 μ g/mL) significantly inhibited the growth of the cancer cells (Supplementary Fig. S3). The sensitivity of each type of cancer cell to rifampicin, as assessed by the concentration required for 50% growth inhibition (GI_{50}), was higher than 4.8×10^{-5} mol/L; the average GI_{50} value was

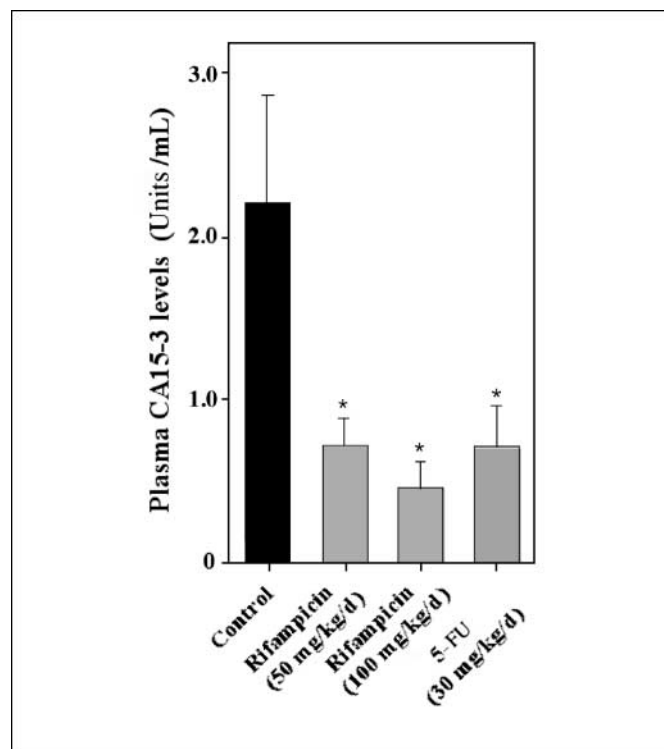


Figure 3. Inhibition of human lung cancer metastasis by p.o. administration of rifampicin. A549 human non-small cell lung cancer cells were injected into the spleen of male immunodeficient mice and the indicated concentrations of rifampicin or 5-FU were administered via a gastric tube for 3 wk. Hepatic metastasis was evaluated by measuring the plasma level of CA15-3, a specific tumor marker produced by A549. Columns, mean for eight mice; bars, SE. *, $P < 0.05$.

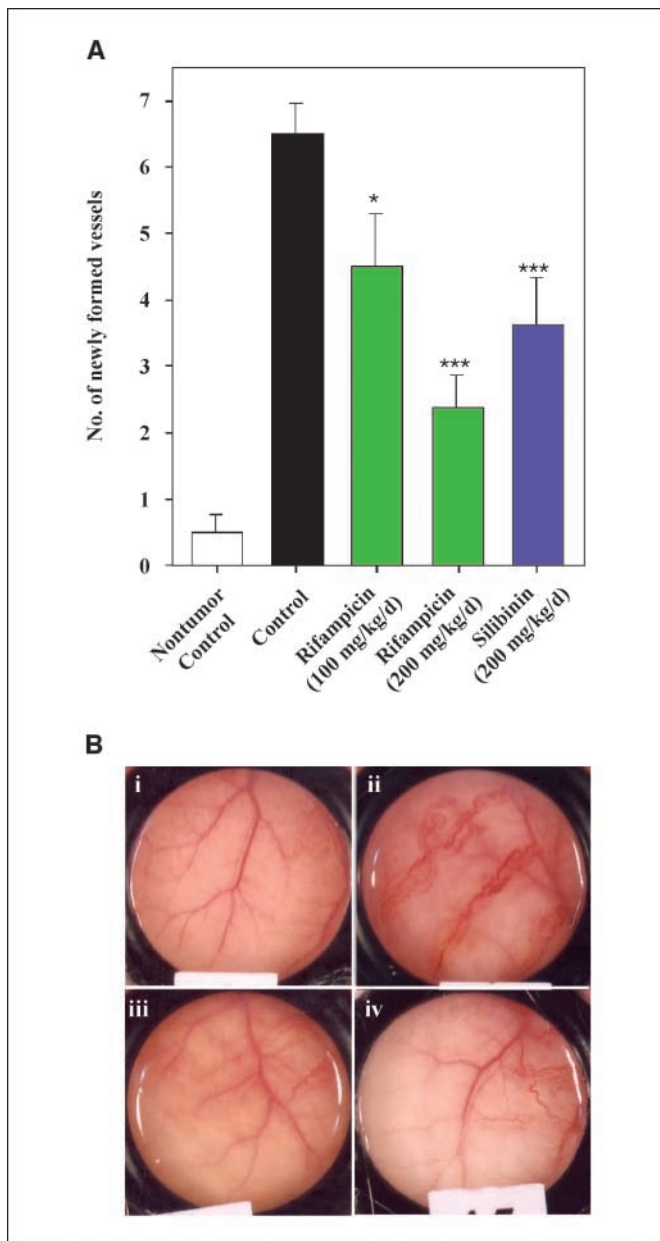


Figure 4. Inhibitory effect of rifampicin on angiogenic response by human sarcoma. Sarcoma 180 cells (A431) were implanted into the mouse dorsal air sac and the indicated doses of rifampicin or silibinin were given via a gastric tube for 5 d. **A**, tumor-induced angiogenesis in the skin was evaluated by counting the number of neovessels microscopically. Columns, mean for eight mice; bars, SE. *, $P < 0.05$; ***, $P < 0.0001$. **B**, morphologic observation of the effect of rifampicin on A431 cell-induced angiogenesis. The area circled with a black ring corresponds to the exact portion of the air sac fascia in contact with a Millipore chamber containing A431 cells. In comparison with nontumor control (i), the air sac fascia of a control mouse was densely capillarized (ii), but very slightly capillarized in the fascia of a mouse treated with 100 mg/kg/d (iii) and 200 mg/kg/d (iv) rifampicin.

$\sim 10^{-4}$ mol/L. Therefore, rifampicin directly suppressed the growth of cancer cells, but the concentrations required for anticancer cell proliferation were higher than those to inhibit endothelial cells.

Rifampicin and 3-formyl rifamycin SV concentration-dependently (0–40 $\mu\text{g}/\text{mL}$) suppressed the progression rate of the denuded edge of monolayer hMVECs, suggesting a migration inhibitory effect (Supplementary Fig. S4A and B). They also inhibited the

migration of hRECs (Supplementary Fig. S4C). However, rifampicin did not acutely block VEGF-induced migration of hUVECs detected by the Boyden chamber method. We next determined whether rifampicin has significant proapoptotic activity. Rifampicin 10 to 40 $\mu\text{g}/\text{mL}$ did not induce apoptosis in rPECs, rAECs, hMVECs, hRECs, or human cancer cells (CW-2, MKN-1, HepG2, U937) when cultured under serum-supplemented conditions. The results showed that rifampicin inhibits anchorage-dependent endothelial cell migration without inducing any appreciable proapoptotic activity in serum-supplemented conditions. Although these features are characteristic of endostatin, the effects of 40 $\mu\text{g}/\text{mL}$ rifampicin on endothelial proliferation seem to be more potent than 10^{-6} mol/L endostatin (8).

We explored whether rifampicin possesses other features characteristic of antiangiogenesis and anticancer reagents. We measured cell-surface ATP synthase activity by quantifying the nucleotides generated in culture media using the CellTiterGlo luminescence assay (30) because potent angiogenesis inhibitors, such as angiostatin, reduce ATP production by binding to the cell-surface ATP synthase β -subunit (31). Rifampicin (10–40 $\mu\text{g}/\text{mL}$) did not suppress ATP production of rAECs incubated at 17% CO_2 and 5% CO_2 , suggesting no effect on ATP synthase activity. The inhibitory effect of rifampicin on histone deacetylase-1 enzymatic activity was measured by quantifying 7-amino-4-methyl coumarin after addition of an acetylated fluorescent peptide to recombinant human histone deacetylase 1 as a substrate. Rifampicin suppressed histone deacetylase 1 activity only at very high concentrations (IC_{50} 375 $\mu\text{g}/\text{mL}$), showing its negligible effect. Rifampicin (0.1–100 $\mu\text{g}/\text{mL}$) did not suppress the infiltration of cultured HT1080 human fibrous sarcoma cells in a Matrigel-based invasion assay (32). Rifampicin at 10^{-5} mol/L (8.2 $\mu\text{g}/\text{mL}$) did not modify nerve growth factor-induced neurite outgrowth of PC12 pheochromocytoma cells, the phenotype associated with colchicine- and Taxol-induced microtubule assembly. Rifampicin (1–100 $\mu\text{g}/\text{mL}$) did not affect the activities of protein kinase A, protein kinase C, epidermal growth factor receptor kinase, or VEGF receptor (Flt-1) kinase (33), whereas it caused 50% to 80% suppression of protein tyrosine kinase, calmodulin-dependent protein kinase III (34), only at 100 $\mu\text{g}/\text{mL}$. Taken together, these results reveal that rifampicin shows some features characteristic of other anticancer reagents but only at high concentrations in the hepatobiliary system.

Down-regulation of angiogenesis-associated genes by rifampicin. We next determined whether rifampicin rapidly down-regulated a variety of growth-, migration-, and angiogenesis-associated genes in the endothelium because this phenomenon is induced by a potent angiogenesis inhibitor, endostatin (8, 9). Using a real-time quantitative RT-PCR, we quantified the mRNA levels of angiogenic factors (VEGF and hepatocyte growth factor), ID1, VEGF receptors (KDR and Flt-1), platelet/endothelial cell adhesion molecule 1, focal adhesion kinase, endothelin 1, endothelin receptor type B, integrin- α_v , integrin- β_3 , and c-myc in hMVECs. Rifampicin rapidly down-regulated all of these genes in a concentration-dependent manner (Fig. 5A); within 4 hours of rifampicin treatment, mRNA levels of each gene treated with 40 $\mu\text{g}/\text{mL}$ rifampicin decreased to levels comparable to those in cells deprived of serum and growth supplements for 4 hours. The ability of rifampicin to down-regulate gene expression is either absent or far less in nonendothelial cells, such as rat fibroblasts and human cancer cell lines (HepG2, MKN-1, and CW-2). Rifamycin SV and 3-formyl rifamycin SV also reduced the mRNA levels of many genes, including integrin- α_v , integrin- β_3 , and c-myc (Fig. 5B); the

spectrum of suppression was almost identical to that of rifampicin. Similar results were obtained with rPECs, rAECs, and hUVECs. These results showed that rifampicin and two rifamycins potently down-regulate proangiogenic gene expression in a variety of endothelial cells, comparable to the effects of endostatin (8).

Discussion

HCV-related liver cirrhosis is a major risk factor for development of HCC, with an annual incidence reaching 7.9% in an untreated, biopsy-proven population (24, 35). Even after initial treatment, second HCCs develop at new foci in ~30% of patients within the first year, leading to a poor prognosis (26). IFN treatment can inhibit HCC in patients with moderate liver fibrosis, but only marginally in liver cirrhosis (35–37). Although the present clinical observation is restricted to only six patients, it should be emphasized that only a single HCC developed during the entire rifampicin therapy period of 97.3 ± 29.1 months despite many features indicating a high risk for HCC (24). First, none had any apparent benefit from IFN therapy; two did not receive IFN; four

never achieved sustained HCV negativity; and three patients discontinued IFN due to adverse reactions. Second, they were relatively old, at 62.0 ± 5.2 years, at the start of rifampicin therapy. Third, they had high serum alanine aminotransferase levels before rifampicin therapy (38). Fourth, patients 1 to 3 showed increasing α -fetoprotein levels when rifampicin was started (39). Patient 1, who had the highest risk for HCC because of relapsing episodes, developed HCC shortly after discontinuation of rifampicin in 1995, but HCC never recurred after restarting rifampicin. Because α -fetoprotein can either be produced by HCC itself or by regenerating hepatic tissues after significant hepatic injury, its marked decrease suggests either reduction of HCC, undetectable by ultrasound or MRI, or suppression of hepatic injury and subsequent regeneration. Whatever the mechanisms, large-scale prospective trials should be done to establish the overall clinical usefulness of low-dose oral rifampicin therapy.

To gain insight into the mechanism behind the clinical benefit of low-dose, long-term rifampicin therapy, we tested its effects on cultured endothelial and nonendothelial cells. Rifampicin and two rifamycins similarly suppressed a variety of mitogenesis- and

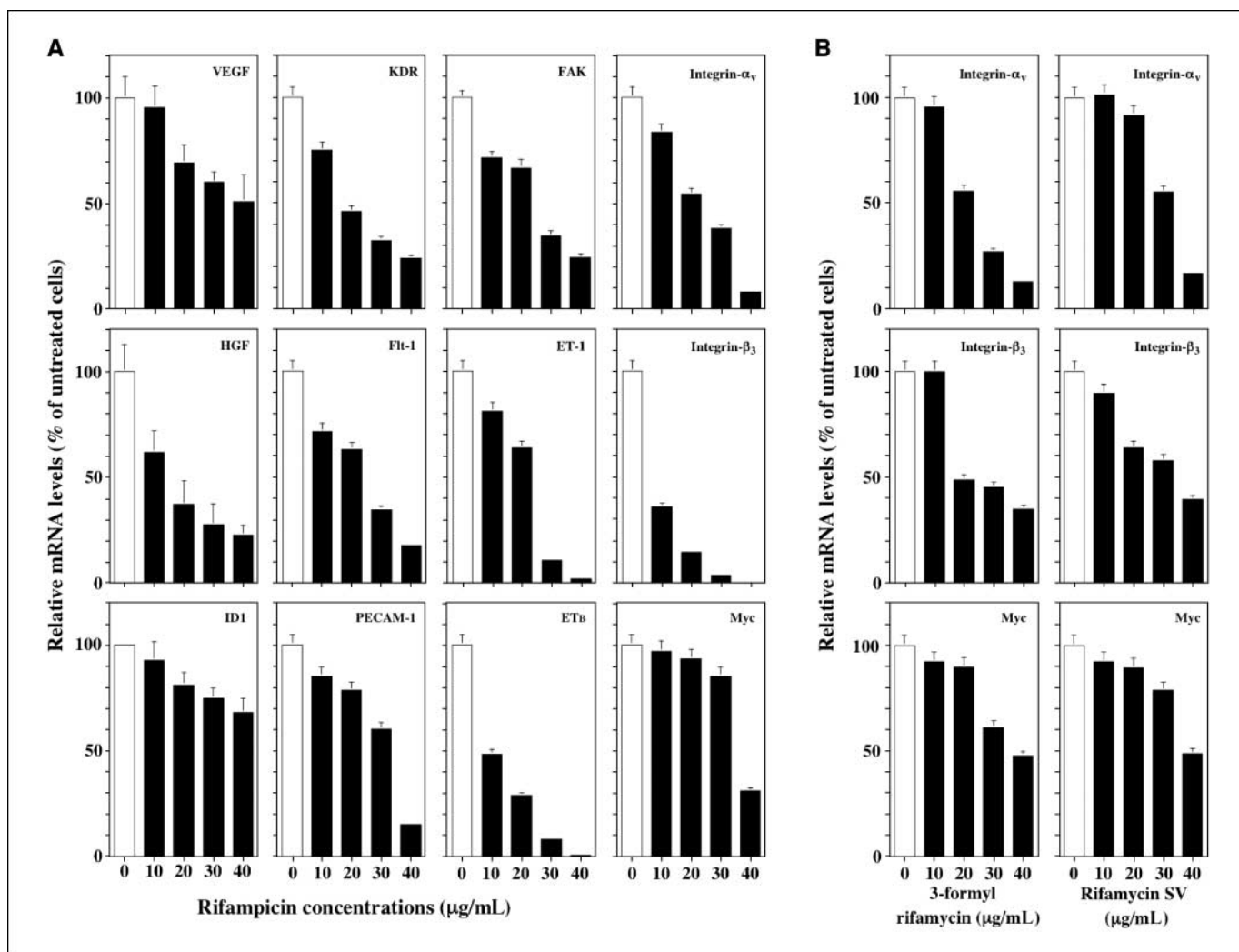


Figure 5. Rifampicin and rifamycins down-regulate growth-, migration-, and angiogenesis-associated genes. hMVECs grown exponentially in 20% FBS and growth supplements were incubated with the indicated concentrations of rifampicin (A), 3-formyl rifamycin SV (B), or rifamycin SV (B) for 4 h and the indicated mRNA levels were determined by real-time quantitative RT-PCR. Columns, mean percentage of the level in untreated cells from four repeated experiments; bars, SE.

angiogenesis-related genes (40) in both human and rat endothelial cells; the time course and potency, as well as the spectrum of gene suppression, are comparable to those previously observed with endostatin (9). There is general agreement that endostatin inhibits cultured endothelial cell migration, but it is less clear whether endostatin also affects endothelial cell apoptosis and proliferation (2, 9, 41–46). In our *in vitro* study using the same protocol as our previous endostatin study (9), the antiproliferative effects of 40 $\mu\text{g}/\text{mL}$ rifampicin and 3-formyl rifamycin SV on microvascular endothelial cells were clearly greater than those seen with the treatment dose of endostatin. An antimigratory effect was also evident, but this was not as marked as the antiproliferative effect. These results show the antiangiogenic properties of rifampicin and 3-formyl rifamycin SV and the potential utility of oral rifampicin as an angiogenesis inhibitor. Considering that its inhibition of endothelial cell proliferation is more potent than that of endostatin, rifampicin could even be used in combination with endostatin, not to mention with other types of angiogenesis inhibitor, or with chemotherapeutic agents. Rifampicin also showed direct growth inhibition of a variety of cancer cells, but only at high concentrations corresponding to bile and urine levels after oral ingestion. The cell growth inhibition profile (20, 22) was similar to that of IFN- α , TZT-1027, and vincristine. These results show that, although rifampicin and 3-formyl rifamycin SV markedly inhibit the proliferation of endothelial cells, the growth-inhibiting effects of rifampicin on cancer cells are less marked, suggesting that rifampicin has a more potent effect through antiangiogenesis rather than a direct cancer-suppressive effect.

For *in vivo* experiments, we used well-established xenograft tumor models in mice to test the effectiveness of rifampicin as an inhibitor of angiogenesis-dependent tumor growth. The growth of CW-2 colon primary tumors was significantly suppressed by oral rifampicin. When rifampicin or rifamycins were administered to Lewis lung carcinoma xenograft mice, lung and liver metastases, as well as tumor growth, were inhibited to a similar extent. Although the doses of rifampicin and rifamycins used in animal studies were >10-fold higher than the doses given to patients, the estimated plasma levels in these animals must be far lower than those showing inhibitory effects on cancer cells. Furthermore, we also performed the mouse dorsal air sac assay, which is well documented to represent tumor-induced angiogenesis and its inhibition in mouse xenografts (47, 48). Thus, significant inhibition of xenograft tumor progression in immunodeficient mice can barely be explained by the direct antitumor effect of rifampicin, but can reasonably be accounted for by its antiangiogenic effect.

Despite the potential use of oral rifampicin as a general angiogenesis inhibitor, its biliary excretion and enterohepatic circulation may be especially beneficial in accumulating this agent and its metabolites in the hepatobiliary system. Biliary and hepatic concentrations of rifampicin and its metabolites are known to be far higher and more sustained than plasma levels after p.o.

administration (49). Peak plasma rifampicin after single oral doses of 150 and 300 mg are as high as 2 and 3.5 $\mu\text{g}/\text{mL}$, respectively, in normal subjects, whereas hepatic bile and urine levels easily exceed 100 $\mu\text{g}/\text{mL}$ (49). Even at 12 hours after an oral dose of 150 mg, hepatic bile concentrations of rifampicin and its metabolite still remain at 100 $\mu\text{g}/\text{mL}$ in normal subjects. In liver cirrhosis, serum and biliary levels are further elevated and mean half-life is prolonged because its bile excretion reaches saturation, resulting in very constant, high levels (50). Such clinical pharmacokinetics of rifampicin explain the remarkable effect of low-dose, once daily, oral rifampicin on suppressing HCC in liver cirrhosis and provide a reasonable rationale for its use as an antiangiogenic agent targeting the hepatobiliary system. Moreover, our results show that, at concentrations >100 $\mu\text{g}/\text{mL}$, rifampicin could exert its direct growth-inhibitory effects on tumor cells, in addition to its antiangiogenic activity, thus allowing an efficient combination of chemotherapy and antiangiogenic therapy by a single oral agent.

In summary, we have shown the effectiveness of oral rifampicin in both clinical and experimental settings. Low-dose, long-term oral rifampicin inhibited the development of HCC in HCV-related liver cirrhosis patients, whereas a comparatively larger dose of oral rifampicin significantly suppressed the growth and metastases of human cancer xenografts in mouse models. In addition, rifampicin exerted antiangiogenic properties. These include down-regulation of proangiogenic genes and inhibition of microvascular endothelial cell proliferation. Rifampicin at higher doses showed direct growth inhibition of cancer cells. Considering its clinical pharmacokinetic profile, oral rifampicin could exert its direct tumor-suppressive effect in the hepatobiliary system and, together with its potent antiangiogenic properties, may be especially beneficial in targeting hepatobiliary tumors.

Disclosure of Potential Conflicts of Interest

M. Shichiri and Y. Tanaka are co-inventors of a patent applied for by the Japan Science and Technology Agency, and M. Shichiri and Y. Kono are co-inventors of a patent applied for by the Tokyo Medical and Dental University. These patents are relevant to this work and are therefore declared as competing financial interests. N. Fukai disclosed no potential conflicts of interest.

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