Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma

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Abstract

In this study, we aimed to evaluate the potential anticancer and antiangiogenic effects of FTY720 on hepatocellular carcinoma. In vitro, chemosensitivity was tested on hepatoma cells, nontumorigenic, immortalized hepatocyte cells, as well as human umbilical vein endothelial cells (HUVEC). Moreover, effect of FTY720 on cell cycle and apoptosis was analyzed. In addition, a number of angiogenesis-associated assays were carried out. The in vivo effect of the drug on hepatocellular carcinoma tumor growth on nude mice was studied. Tissues obtained were analyzed in terms of proliferation, apoptosis, tumor microvessel density, and tumor vascular permeability. Compared with the MIHA cells, the hepatoma cell lines as well as HUVECs were found to be highly sensitive to the drugs in the aspect that FTY720 could induce G1 arrest and apoptosis in the hepatoma cells. Furthermore, FTY720 significantly decreased invasion, migration, and capillary tube formation of HUVECs at very low doses. In vivo study showed that tumor growth was significantly suppressed in the FTY720-treated animals, and staining of the tissue sections showed decreased tumor cell proliferation and increased tumor cell apoptosis in the treatment groups. Interestingly, significant reductions in tumor microvessel density and tumor vascular permeability were also found in the FTY720-treated groups. In conclusion, FTY720 not only shows potent antiangiogenic effects but is also cytotoxic toward hepatoma cells. Results from our preclinical study suggest that FTY720 can be selected as a good candidate for the treatment of hepatocellular carcinoma. [Mol Cancer Ther 2005;4(9):1430–8]

Introduction

Hepatocellular carcinoma is one of the most common malignancies worldwide. Being most prevalent in Asia and Africa, it is the second leading cause of cancer death in China. Whereas tumor resection and liver transplantation provide a chance of cure for hepatocellular carcinoma, the postoperative recurrence rate is high (1, 2). Furthermore, most patients with hepatocellular carcinoma present with advanced tumors which are not amenable to surgical treatment, and, currently, there is no effective systemic chemotherapy for this cancer. Hence, the search for novel therapies for hepatocellular carcinoma is of paramount importance.

With the recent advances in the understanding of cancer growth mechanisms, it is clear that the therapeutic targets for an anticancer therapy include not only the tumor cells themselves but also the tumor matrix, stroma, and vasculature. Hepatocellular carcinoma is a tumor characterized by active neovascularization. Our previous studies have shown that angiogenesis plays an important role in the progression of hepatocellular carcinoma. In particular, the presence of microvessels and the expression of vascular endothelial growth factor have been significantly implicated in vascular invasion and tumor recurrence (3, 4). Antiangiogenesis represents a novel therapeutic strategy that may be effective in the treatment of hepatocellular carcinoma.

FTY720 is derived from ISP-1 (myriocin), which is a fungal metabolite known as an eternal youth nostrum in traditional Chinese herbal medicine (5). It was originally derived as a novel immunomodulatory drug for use in organ transplantation to prolong graft survival (6). It was shown to induce apoptosis of infiltrated lymphocytes, especially T cells, for prolongation of allograft survival after solid organ transplantation (7, 8). Recently, FTY720 has been reported to have a strong antitumor effect on breast cancer, bladder cancer, and leukemia (9–11). The drug was found to induce apoptosis in the human prostate cancer cell line DU145 (12). Meanwhile, it was reported that FTY720 induced G0/G1 arrest in HL60RG and Jurkat cells via dephosphorylation of retinoblastoma protein (13). Interestingly, FTY720 was found to become biologically activated by phosphorylation into FTY720-P (14, 15), which is a novel high-affinity agonist for sphingosine-1-phosphate receptors (16, 17) that are widely expressed on endothelial cells. Furthermore, FTY720-P has been shown to affect endothelial cell functions, suggesting
that FTY720 might exert a biological effect on the vasculature via its active form, FTY720-P (18, 19). However, its effect on tumor-associated endothelial cells, and thus tumor angiogenesis, has not yet been explored.

The therapeutic potential of FTY720 in hepatocellular carcinoma has not been studied thus far, and its effect on tumor angiogenesis is still unknown. Hence, in this study, we evaluated both the in vitro and in vivo effects of FTY720 on angiogenesis and hepatocellular carcinoma tumor growth.

Materials and Methods

Cell Lines and Culture Conditions

The human hepatocellular carcinoma cell lines PLC/PRF/5 and Huh7 (20) and the human nontumorigenic, immortalized liver cell line MIHA (21) were obtained and maintained as previously described (22). The human umbilical vein endothelial cells (HUVEC; Cascade Biologies, Portland, OR) were grown in complete endothelial growth medium and used between passages 4 and 6 only.

Drug

FTY720 was kindly provided in powder form by Novartis Pharmaceuticals Ltd. (Basel, Switzerland). It was used after dissolving the powder in normal saline.

In vitro Proliferation Assay

The hepatocellular carcinoma cell lines, nontumorigenic, immortalized hepatocyte cell line MIHA, and HUVECs were seeded onto 96-well plates, and appropriate concentrations of FTY720 ranging from 0.078 to 100 μmol/L were then added. After 24 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done as described previously (22).

Cell Invasion Assay

The ability of HUVECs to invade through a basement membrane was assessed using BD BioCoat Matrigel Invasion Chambers (BD Biosciences Discovery Labware, Bedford, MA) according to the recommendations of the manufacturer. The amount of cells that invaded to the other side of the membrane 24 hours after seeding was first stained with crystal violet, and followed by elution with 10% acetic acid for absorbance reading at 540 nm. Duplicate chamber inserts were done in each of the three independent experiments.

Wound Migration Assay

HUVECs were seeded in 96-well cell culture plates and allowed to grow to 70% to 80% confluency. A change of fresh medium was added either in the absence of FTY720 to serve as control or with FTY720 at 3.0 μmol/L. Triplicates of wells were done for each condition in a single experiment. A scrape was made with a sterile tip to create a gap with uniform width in all wells containing cell monolayers. The wounds were then photographed under phase-contrast microscope at a final magnification of ×200. The cells were incubated for 24 hours before the wounded locations were photographed again for the assessment of cell migration across the gap. At least three independent experiments were carried out to confirm the results.

Capillary Tube Formation

Endothelial cells form hollow tubelike structure on plating in matrigel. Cell culture plates were coated with Matrigel (BD Biosciences Discovery Labware) according to the recommendations of the manufacturer. After seeding the HUVECs with or without FTY720 treatment, tube formation was observed periodically over a 24-hour period under a phase-contrast microscope. The experiments were repeated at least thrice.

Cell Cycle Analysis

PLC/PRF/5 cells, 5 × 10^4, were seeded per well in six-well plates and were allowed to grow to 70% to 80% confluency before FTY720 was added to the culture medium. Cells were harvested after 24 hours and fixed in 70% ice-cold ethanol. Before fluorescence-activated cell sorting analysis, the fixed cells were washed in PBS, treated with RNase (1 μg/mL), and stained with propidium iodide (50 μg/mL) for 30 minutes at 37°C. DNA content and cell cycle distribution of PLC/PRF/5 cells were analyzed in an EPICS profile analyzer using ModFit LT2.0 software (Coulter Electronics, Hialeah, FL).

Apoptosis Analysis

For cells grown in culture, caspase-3 activity was measured using a caspase-3 colorimetric detection kit (Chemicon International, Inc., Temecula, CA). In brief, PLC/PRF/5 and Huh7 cells were treated with FTY720 at IC_{10} and IC_{50} dosages and harvested at 24 hours. Cells lysates were incubated with caspase-3 substrate solutions before absorbance readings were obtained at 405 nm. The amount of caspase-3 enzyme activity was extrapolated from a standard curve. Each treatment was done in duplicates, and at least three individual experiments were carried out. For analysis of cells grown on chamber slides (cytospin was done before analysis) and the tissue sections, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining was done using the In situ Cell Death Detection Kits according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany). Cells with TUNEL-positive nuclei were scored as apoptotic cells. The images were captured by an image analysis system (Eclipse E600, Nikon, Tokyo, Japan).

Tumor Xenograft Experiments

The xenografts were established using the hepatocellular carcinoma cell lines PLC/PRF/5 and Huh7. The animals used were 4- to 6-week-old male athymic nude mice (BALB/c-nu/nu). At least six serial passages had been done on the animals before the use of this hepatocellular carcinoma xenograft model in the FTY720 study. Treatment was started once the size of the xenograft reached around 4 × 4 mm (length × width) in size, during which the mice were randomly assigned into the treatment and control (vehicle only) groups. For the treatment group, FTY720 was administered by daily i.p. injection of 10 mg/kg/d for 20 days. The mice in both the treatment and control groups (n = 15 in each group) were sacrificed on day 21 when snap-frozen and paraffin-embedded tumor tissue blocks had been obtained for further analysis. The body weight and tumor size were recorded twice a week starting from...
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the day of treatment. The tumor size was measured by a caliper, and the tumor volume was calculated using the equation length × width² × 0.5. The study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

Histology and Immunohistochemistry
Paraffin-embedded, 4-μm sections were deparaffinized, rehydrated, and subjected to H&E staining or immunohistochemical staining. Evaluation of tumor microvessel density was done by anti-mouse CD34 monoclonal antibody staining (clone MEC14.7, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Cell proliferation analysis was done by anti-mouse proliferating cell nuclear antigen (PCNA) monoclonal antibody staining (clone PC10, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The standard avidin-biotin-peroxidase complex technique was employed. The negative control was obtained by substituting the primary antibodies with mouse immunoglobulin G.

Quantification of Microvessel Density and Tumor Cell Proliferation
The microvessel density of the xenograft tissue sections was evaluated according to Gasparini’s criteria (23). At low power field (40×), areas with the most intense neo-vascularization (hotspots) on the section were selected. Microvessel counts of these areas were done at high power field (200×). The mean microvessel count of at least three most vascular areas was calculated as microvessel density for each section. The mean microvessel density from five animals in the treatment and control groups was obtained as the final microvessel density count. The microvessel density was expressed as the absolute number of microvessels per 0.74 mm² (200× field). Tumor cell proliferation was evaluated by counting the positively stained nuclei of the tumor cells at 400× field after PCNA staining. It was then expressed as the percentage over the total number of tumor cells presented in the field. At least five fields were counted for each sample section, and the mean scores from five samples in each of the treatment and control groups were recorded.

Vascular Permeability Analysis
Modified Miles assay (24, 25) was used to test the tumor vessel permeability based on the principle that a complex between the sulfonic acid group of Evans blue and the terminal cationic nitrogens of the lysine residue in albumin can be formed by electrostatic combination (26, 27). Evans blue was dissolved in physiologic saline (1 mg/mL) and administered into tumor-bearing nude mice by i.v. injection at a dose of 10 μL/g of body weight. After 15 minutes, the mice were perfused with 25 mL of physiologic saline through the left ventricle of the heart. The tumor was excised, cut into small pieces (avoiding the necrotic areas), weighed, and put in 50% trichloacetic acid (w/v) for extraction of the dye (10 μL/mg of tumor tissue). The concentrations of the dye were determined by measuring the light absorbance at 620 nm.

Electron Microscopy
After 10 days of FTY720 treatment, tumor tissues from the treated and control mice were taken. The tissues were fixed immediately in 2.5% glutaraldehyde in cacodylate buffer (sodium cacodylate-hydrochloric acid buffer 0.1 mol/L, pH 7.4) overnight at 4°C for electron microscopy sectioning. The tissue sections were examined with a transmission electron microscope (EM208S; Philips, Eindhoven, Holland).

Statistical Analysis
Unpaired Student’s t test was used to analyze the differences in terms of growth rate, PCNA scores, microvessel density counts, and vascular permeability readings between the treatment and control groups. Statistical significance was taken as P < 0.05. All statistical analyses were done using the SPSS statistical software (SPSS Inc., Chicago, IL).

Results
FTY720 Selectively Inhibited Hepatoma Cell Proliferation and Induced G1 Cell Cycle Arrest and Apoptosis in Hepatoma Cells
The specific antitumor cell effect of FTY720 was first shown by in vitro cell proliferation analysis using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 1). Our result revealed that the cells are sensitive to the drug in a dose-dependent manner. The graph (see also Table 1) illustrated that at 24 hours following drug treatment, FTY720 had a cytotoxic effect on PLC/PRF/5

Table 1. Comparison of cytotoxic effects of FTY720 on different cells

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<th>IC50 at 24 h (μmol/L)</th>
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Figure 1. In vitro cytotoxic effect of FTY720 on different cells: selective inhibition of tumor cell and HUVEC proliferation. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed the chemosensitivity of PLC/PRF/5, Huh7, and MIHA cell lines and HUVECs at 24 h post FTY720 treatment. FTY720 treatment led to decreased cell proliferation in PLC/PRF/5, Huh7, and HUVECs in a dose-dependent manner. Points, mean; bars, SD. Representative of at least three independent experiments.
Figure 2. In vitro analysis of cell cycle and apoptosis. A, G1 arrest of tumor cells; fluorescence-activated cell sorting analysis of PLC/PRF/5 and Huh7 cells at 0 and 24 h post FTY720 treatment at indicated dosages. It is shown here that while there was no significant change in percentage of cells in G1 content for the control cells at 0 and 24 h, G1 arrest is shown in a dose-dependent manner in the FTY720-treated cells. B, induction of tumor cell apoptosis by TUNEL staining. The percentage of sub-G1 phase cells was analyzed by TUNEL assay. PLC/PRF/5 and Huh7 cells that showed nuclear fragmentation were considered to be undergoing apoptosis. Apoptotic cells increased in a dose-dependent manner. Results were derived from three independent experiments; bars, SE. C, induction of tumor cell apoptosis by measuring relative caspase-3 activity. Effect of FTY720 on induction of hepatoma cell apoptosis is shown by increased expression of caspase-3 activity in the treatment group as compared with control by colorimetric reading at 405 nm. The caspase-3 activity in the control group was taken as 100%; data obtained from other treatment points were expressed relative to the control.
and Huh7 cell lines with IC_{50} of 10 and 12 μmol/L, respectively; in contrast, the nontumorigenic hepatocytes, MIHA, had much higher chemoresistance to the drug with IC_{50} of 60 μmol/L at 24 hours post drug treatment, suggesting that FTY720 could selectively inhibit hepatoma cell proliferation with little effect on normal hepatocytes.

To have a clearer picture of the antiproliferative activity of FTY720 on the hepatoma cells, we analyzed the drug effect on cell cycle regulation and cell apoptosis by assessing DNA content and DNA strand breaks, respectively, as described in Materials and Methods. For the DNA content analysis, PLC/PRF/5 and Huh7 cell lines treated with or without FTY720 were analyzed by fluorescence-activated cell sorting at 0 and 24 hours using IC_{10} and IC_{50} dosages with respect to the 24-hour drug treatment. Figure 2A shows that G1 arrest was exhibited in the FTY720-treated cells. For example, PLC/PRF/5 cells treated with drug showed that G1 content increased to 65.0% (at IC_{10} dosage) and 69.5% (at IC_{50} dosage), respectively, compared with 49.6% in the control. It is known that cell detachment is an early event in apoptosis. Because only adherent cells were collected and cell medium was discarded in our cell cycle analysis, further assays were done for analysis of apoptosis. By TUNEL assay, cells that were TUNEL positive were increased after FTY720 treatment, as shown in Fig. 2B. For instance, PLC/PRF/5 cells treated with drug had 8.4% (at IC_{10} dosage) and 48.2% (at IC_{50} dosage) TUNEL-positive cells, respectively, compared with only 1.1% in the control cells. Moreover, Fig. 2C shows that relative caspase-3 activity was also increased in the FTY720-treated cells; in particular, PLC/PRF/5 cells treated with drug had 1.6-fold (at IC_{10} dosage) and 6.9-fold (at IC_{50} dosage) increases in caspase-3 activity compared with control cells. Therefore, our in vitro data suggest that FTY720 is cytotoxic to the hepatoma cells and that it could induce G1 arrest and cell apoptosis in a dose-dependent manner.

**FTY720 Inhibited Proliferation, Invasion, Migration, and Capillary Tube Formation of HUVECs**

The antiangiogenic potential of FTY720 was examined using HUVECs. As shown in Fig. 1, FTY720 has a dramatic growth inhibitory effect on the HUVECs at much lower dose levels as compared with the hepatoma cells (IC_{50} at 24 hours was 10 μmol/L for PLC/PRF/5 cells and MIHA cells; IC_{50} at 24 hours was 60 μmol/L), in that IC_{50} at 24 hours was around 4.8 μmol/L, implying that FTY720 could exert an even greater antiproliferative effect on endothelial cells than on hepatoma cells.

We next examined the effect of FTY720 on endothelial cell movement through extracellular matrix by a cell invasion assay as described in Materials and Methods. As shown in Fig. 3A, 24 hours after seeding the HUVECs suspended in basal medium in the upper part of the insert in the absence or presence of FTY720, cells that invaded across the extracellular matrix and migrated to the lower part of the insert were photographed and quantified. With the extent of cell invasion in the absence of FTY720 taken as 100%, our result showed that FTY720 at 1.5 μmol/L could significantly decrease cell invasion to 44%, whereas at 3 μmol/L, cell invasion was dramatically reduced to only

![Image](80x298 to 288x383)

**Figure 3.** In vitro inhibitory effect of FTY720 on HUVECs. A, inhibition of HUVEC invasion. HUVECs suspended in basal endothelial cell growth medium with 0, 1.5, or 3.0 μmol/L of FTY720 were seeded on the thin coat of Matrigel layer of the cell culture insert and incubated at 37 °C for 24 h. Cells that invaded the Matrigel and migrated to the lower surface of the insert were stained with crystal violet; representative photos were taken at ×200 magnification before the stains taken by the cells were dissolved in 10% acetic acid for absorbance reading. Each treatment was done in triplicate, and at least two independent experiments were carried out. Cells that invaded the Matrigel and migrated to the lower surface of the insert were quantified by colorimetric reading for the amount of stains that were taken up by the cells on the lower surface. In brief, the readings obtained from controls (0 μmol/L FTY720) were taken as 100%; data (see histograms) obtained from the FTY720-treated groups were expressed as percentages relative to the control. Columns, mean percentage from two independent experiments; bars, SD. *, P < 0.05, compared with control. B, inhibition of HUVEC migration. Monolayers of HUVECs were wounded with sterile pipette tips and the cells were incubated without FTY720 (control) or with 3 μmol/L of FTY720 for 24 h. Representative photos (×200) are shown for the wound areas before and 24 h after FTY720 treatment (3 μmol/L) as compared with control. C, effect on capillary tube formation. HUVECs suspended in complete endothelial growth medium with or without addition of FTY720 were seeded on thin layers of Matrigel and incubated at 37 °C. Extent of capillary tube formation was monitored at time intervals over a 24-h period. Representative photos (×200) shown here illustrate the effect of FTY720 on capillary tube formation at 4 and 24 h posttreatment.
18%. To further complement the invasion data, wound migration assay was then carried out to examine the effect of FTY720 on cell migration. As shown in Fig. 3B, when the monolayer of confluent culture of endothelial cells was wounded and incubated in fresh complete medium in the absence or presence of FTY720 for 24 hours, the wound was clearly unable to heal after 24 hours when 3 A mol/L of FTY720 was present in the medium. Our results therefore indicate that FTY720 could prevent endothelial cell invasion and migration at low doses.

To further examine the effect of FTY720 on endothelial cells, we carried out capillary tube formation assay to investigate if FTY720 affected the endothelial cell interaction with extracellular matrix. By seeding the HUVECs suspended in complete medium with or without FTY720 onto a Matrigel layer, the process of capillary tube formation was monitored and photographed at 4 and 24 hours postincubation. We observed that at 4 hours, the presence of FTY720 seemed to slow down the process of capillary tube formation in a dose-dependent manner, whereas at 24 hours, the impaired elongation and alignment of the cells could be observed with cells incubated in the presence of FTY720 (Fig. 3C).

Taken together, our in vitro assays have shown that FTY720 could inhibit proliferation of endothelial cells as well as affect the invasion, migration, and capillary tube formation of endothelial cells. Considering that endothelial cell proliferation, invasion, migration, and tube formation are all highly relevant properties involved in the process of angiogenesis, our data therefore showed strong in vitro evidence for the possible antiangiogenic potential of FTY720 in vivo.

**FTY720 Suppressed Tumor Growth, Inhibited Tumor Cell Proliferation, and Induced Tumor Cell Apoptosis In vivo**

We next examined the effect of FTY720 on the in vivo nude mice models bearing PLC/PRF/5- and Huh7-derived hepatocellular carcinoma xenografts. We studied the drug effect on the established xenografts by allowing the tumor implant to grow to about 4 x 4 mm (length x width) in size before the mice were randomly divided into the treatment and control groups. To obtain sufficient tumor tissues after subjecting the animals to a maximum drug treatment without inducing marked toxicity, we chose to use daily injection of 10 mg/kg/d of FTY720 on the already clearly established tumors for the entire study. The animals were sacrificed on day 21. Those mice that were treated with FTY720 showed significantly suppressed tumor growth (Fig. 4A and B; \( P < 0.001 \) on day 20 for both cell lines tested). No significant weight loss (Fig. 4C) or adverse effects on the general well-being were observed among the animals.
On dissecting the tumor tissues, the macroscopic and microscopic appearances of the tumor tissues were examined. Hepatocellular carcinoma is known to be a tumor with rich vasculature, and among the two hepatocellular carcinoma cell lines that we had implanted in the nude mice, Huh7-derived xenografts were richer in blood vessels ("vascular") by its macroscopic appearance. When the tumors were dissected, the Huh7 xenografts from the treatment group appeared less vascular than the control group (Fig. 4A). Further H&E examination showed that there was a greater degree of tumor necrosis in the treatment group as compared with the control group (Fig. 5A, a and b). On histologic examination by staining for cell proliferation (PCNA) and apoptosis (TUNEL assay), cell apoptosis was increased in all FTY720-treated xenografts, whereas cellular proliferation was significantly decreased in the FTY720-treated group (Fig. 5A and B).

FTY720 Reduced Tumor Microvessel Density and Decreased Vascular Permeability in the Tumor Tissues

Comparison of the microvessel densities of the tumors in the treatment and control groups showed significantly reduced microvessel density in all tumor tissues from the FTY720-treated group (Fig. 6A). However, a more dramatic decrease in microvessel density by FTY720 treatment was observed in the xenografts derived from the PLC/PRF/5 cell line than those derived from the Huh7 cell line.

We next verified the ultrastructural microvessel integrity in the treatment and control groups by examination under the electron microscopy. The photos illustrated in Fig. 6B show that in the control group (a), the microvessels showed gaps between the vessel lining endothelial cells, and that the vessel lining was irregularly organized with very thin cell-to-cell contact. In contrast, in the treatment group (Fig. 6B, b), the vascular lining was more tightly assembled.

**Figure 5.** *In vivo* effect of FTY720 on hepatocellular carcinoma cells. A, histologic comparison between control (top) and treated (10 mg/kg/d; bottom) tissue sections: H&E staining (a and b, ×100); PCNA staining (c and d, ×400); and TUNEL staining (e and f, ×400). The photos represent the PLC/PRF/5-derived tumors. B, quantitative comparison by PCNA scores between control (n = 5) and treatment (10 mg/kg/d) groups (n = 5) in PLC/PRF/5- (P < 0.05) and Huh7- (P < 0.05) derived xenografts. *, P < 0.05, compared with control.
In this study, we showed for the first time that FTY720 could effectively suppress hepatocellular carcinoma tumor growth both in vitro and in vivo. We have shown here that tumor cell proliferation was significantly suppressed by FTY720 treatment both in vitro and in vivo. Moreover, as shown by in vitro and in vivo analyses of hepatoma cell apoptosis, it was clear that one of the antitumor actions of FTY720 is likely to be via induction of tumor cell apoptosis. In the literature, there have been reports of in vitro studies demonstrating the possible molecular mechanisms involved in the anticancer action of FTY720 in breast, bladder, and prostate cancer cell lines (9, 10, 12). From these in vitro studies, it is understood that FTY720 could have a suppressive effect on tumor cell growth by inducing tumor cell apoptosis, decreasing tumor cell proliferation, and reducing actin stress fiber formation in the cancer cells (9, 10, 12). Taken together, we can conclude that FTY720 inhibits hepatocellular carcinoma tumor growth, at least partly, by selectively suppressing tumor cell proliferation and inducing tumor cell apoptosis. Therefore, our data provide positive evidence that FTY720 has the potential to be used as an anticancer agent for hepatocellular carcinoma.

From the recent literature, it was suggested that FTY720 might have a modulatory effect on angiogenesis (18, 19). However, its antiangiogenic effect has not been reported thus far. Hepatocellular carcinoma is known to be a tumor with rich vasculature, and angiogenesis plays important roles in its development, growth, and progression (3, 28). Hence, after we have confirmed that FTY720 could suppress growth of hepatocellular carcinoma, we hypothesized that such suppression of tumor growth might not only be attributed to the direct cytotoxic actions of FTY720 on the tumor cells but might also be partly due to the possible modulatory effect of FTY720 on the tumor vasculature. Indeed, our in vitro results on the study of HUVECs showed strong evidence that FTY720 has potent antiangiogenic properties. Moreover, the significant reduction in tumor microvessel density in the treatment group from our animal study provided the first piece of in vivo evidence that FTY720 influences tumor angiogenesis.

It is known that “leaky vessels” are one of the characteristics of tumor neovasculature that make it different from the normal vasculature (29–31). We therefore investigated the possible modulatory effect of FTY720 on tumor vessel permeability. First, the ultrastructural images from electron microscopic sections confirmed dramatic differences as revealed by the fact that the assembly of the lining endothelial cells was much more adhered and tightened in the FTY720-treated group, indicating the decreased permeability of the vessels in the FTY720-treated tumors. Furthermore, results from the modified Miles assay showed that the total vascular permeability in the tumor xenograft was significantly reduced in the FTY720-treated group. Although microvessel density was reduced in the treated group of PLC/PRF/5-derived tumors, the macroscopic appearance (Fig. 4A) as well as microscopic examination of the sections (Fig. 6B) suggested that bigger vessels, rather than microvessels, were still present in the FTY720-treated PLC/PRF/5-derived tumors. Therefore, whereas the reduction of permeability could be partly ascribed to the overall reduced microvessel density of the tumor tissues of the FTY720-treated group, it could also reflect the reduced leakiness of the bigger vessels that were still present in the treated tumors. In support of our findings, our results corroborated with much greater cell-to-cell contact. By further performing the modified Miles assay, we observed a statistically significant reduction in vascular permeability in the tumor tissues from the treatment group compared with the control group (Fig. 6C).

**Discussion**

In this study, we showed for the first time that FTY720 could effectively suppress hepatocellular carcinoma tumor growth both in vitro and in vivo. We have shown here that tumor cell proliferation was significantly suppressed by FTY720 treatment both in vitro and in vivo. Moreover, as shown by in vitro and in vivo analyses of hepatoma cell apoptosis, it was clear that one of the antitumor actions of FTY720 is likely to be via induction of tumor cell apoptosis. In the literature, there have been reports of in vitro studies demonstrating the possible molecular mechanisms involved in the anticancer action of FTY720 in breast, bladder, and prostate cancer cell lines (9, 10, 12). From these in vitro studies, it is understood that FTY720 could have a suppressive effect on tumor cell growth by inducing tumor cell apoptosis, decreasing tumor cell proliferation, and reducing actin stress fiber formation in the cancer cells (9, 10, 12). Taken together, we can conclude that FTY720 inhibits hepatocellular carcinoma tumor growth, at least partly, by selectively suppressing tumor cell proliferation and inducing tumor cell apoptosis. Therefore, our data provide positive evidence that FTY720 has the potential to be used as an anticancer agent for hepatocellular carcinoma.

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**Discussion**

In this study, we showed for the first time that FTY720 could effectively suppress hepatocellular carcinoma tumor growth both in vitro and in vivo. We have shown here that tumor cell proliferation was significantly suppressed by FTY720 treatment both in vitro and in vivo. Moreover, as shown by in vitro and in vivo analyses of hepatoma cell apoptosis, it was clear that one of the antitumor actions of FTY720 is likely to be via induction of tumor cell apoptosis. In the literature, there have been reports of in vitro studies demonstrating the possible molecular mechanisms involved in the anticancer action of FTY720 in breast, bladder, and prostate cancer cell lines (9, 10, 12). From these in vitro studies, it is understood that FTY720 could have a suppressive effect on tumor cell growth by inducing tumor cell apoptosis, decreasing tumor cell proliferation, and reducing actin stress fiber formation in the cancer cells (9, 10, 12). Taken together, we can conclude that FTY720 inhibits hepatocellular carcinoma tumor growth, at least partly, by selectively suppressing tumor cell proliferation and inducing tumor cell apoptosis. Therefore, our data provide positive evidence that FTY720 has the potential to be used as an anticancer agent for hepatocellular carcinoma.

From the recent literature, it was suggested that FTY720 might have a modulatory effect on angiogenesis (18, 19). However, its antiangiogenic effect has not been reported thus far. Hepatocellular carcinoma is known to be a tumor with rich vasculature, and angiogenesis plays important roles in its development, growth, and progression (3, 28). Hence, after we have confirmed that FTY720 could suppress growth of hepatocellular carcinoma, we hypothesized that such suppression of tumor growth might not only be attributed to the direct cytotoxic actions of FTY720 on the tumor cells but might also be partly due to the possible modulatory effect of FTY720 on the tumor vasculature. Indeed, our in vitro results on the study of HUVECs showed strong evidence that FTY720 has potent antiangiogenic properties. Moreover, the significant reduction in tumor microvessel density in the treatment group from our animal study provided the first piece of in vivo evidence that FTY720 influences tumor angiogenesis.

It is known that “leaky vessels” are one of the characteristics of tumor neovasculature that make it different from the normal vasculature (29–31). We therefore investigated the possible modulatory effect of FTY720 on tumor vessel permeability. First, the ultrastructural images from electron microscopic sections confirmed dramatic differences as revealed by the fact that the assembly of the lining endothelial cells was much more adhered and tightened in the FTY720-treated group, indicating the decreased permeability of the vessels in the FTY720-treated tumors. Furthermore, results from the modified Miles assay showed that the total vascular permeability in the tumor xenograft was significantly reduced in the FTY720-treated group. Although microvessel density was reduced in the treated group of PLC/PRF/5-derived tumors, the macroscopic appearance (Fig. 4A) as well as microscopic examination of the sections (Fig. 6B) suggested that bigger vessels, rather than microvessels, were still present in the FTY720-treated PLC/PRF/5-derived tumors. Therefore, whereas the reduction of permeability could be partly ascribed to the overall reduced microvessel density of the tumor tissues of the FTY720-treated group, it could also reflect the reduced leakiness of the bigger vessels that were still present in the treated tumors. In support of our findings, our results corroborated with much greater cell-to-cell contact. By further performing the modified Miles assay, we observed a statistically significant reduction in vascular permeability in the tumor tissues from the treatment group compared with the control group (Fig. 6C).
with and complemented the experiments done with FTY720 in normal tissues by Sanchez et al. (18). We are the first group to show in vivo in the tumor tissues that FTY720 treatment could reduce tumor vessel permeability.

FTY720 is currently being evaluated in clinical trials as a novel immunomodulating agent in the prolongation of graft survival after organ transplantation (32). In this context, FTY720 may be particularly suitable to be used after liver transplantation for hepatocellular carcinoma. In fact, we have shown in a rat liver transplantation model that FTY720 could effectively attenuate liver injury (33). In addition to its effect on prevention of graft rejection and injury, FTY720 may confer an antitumor effect and benefit to the patients by reducing the chance of growth and metastasis. It would be worthwhile to investigate the effect of FTY720 on tumor recurrence after liver transplantation for hepatocellular carcinoma. Currently, there is neither an effective adjuvant treatment that has been shown to be effective in preventing tumor recurrence after transplantation for hepatocellular carcinoma, nor a systemic therapy that has been proven to be potent for the treatment of hepatocellular carcinoma. FTY720 could also be a potential new treatment for advanced or metastatic hepatocellular carcinoma.

In conclusion, this study shows for the first time that FTY720 is a potent antivascular agent, and that it is effective in inhibiting growth of hepatocellular carcinoma. We have shown that, in addition to its direct antitumor activity through antiproliferative and apoptosis-inducing effects, FTY720 could also inhibit tumor growth by modulating the tumor vasculature. Our study is the first to report that FTY720 treatment has an antiangiogenic effect on tumor vasculature. Further functional studies are required to unfold the underlying mechanisms through which FTY720 modulates angiogenesis. FTY720 may provide a novel therapeutic strategy for hepatocellular carcinoma through its antiproliferative and antiangiogenic effects.

References


Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma

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