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Antiproliferative and Overadditive Effects of Rapamycin and FTY720 in Pancreatic Cancer Cells In Vitro

Y. Shen, X. Wang, W. Xia, C. Wang, M. Cai, H. Xie, L. Zhou, and S. Zheng

ABSTRACT

Rapamycin inhibits the growth of several tumors including pancreatic carcinoma both in vitro and in vivo. The antitumor effects of FTY720 were also shown recently. The present study was performed to investigate the in vitro antiproliferative capacity of combined treatment with rapamycin and FTY720 on pancreatic cacinoma cell lines.

Materials and Methods. The Panc-1 and AsPc-1 cell lines were employed as the pancreatic carcinoma model in vitro. For monotreatment experiments, rapamycin was added in increasing doses from 0.002 μmol/L to 200 μmol/L; FTY720 was used from 1 μmol/L to 15 μmol/L. For combined treatment, two concentrations of rapamycin were combined with seven concentrations of FTY720; or two concentrations of FTY720 with five concentrations of rapamycin. The antiproliferative capacity was assessed by the MTT assay.

Results. Rapamycin and FTY720 inhibited MTT incorporation into Panc-1 and AsPc-1 in dose-dependent fashion with or without serum stimulation. In coincubation experiments, great susceptibility to rapamycin was seen when combined with 10 μmol/L FTY720. An effective combination for AsPc-1 was 10 μmol/L FTY720 with 0.002 μmol/L rapamycin, resulting in more than 50% inhibition of MTT incorporation, and for Panc-1, 10 μmol/L FTY720 with 0.002 μmol/L rapamycin and 10 μmol/L FTY720 with 20 μmol/L rapamycin; the corresponding inhibition levels reached about 40% and 60%, respectively.

Conclusion. Rapamycin and FTY720 showed dose-dependent antiproliferative effects on pancreatic carcinoma cell lines in vitro both alone and in combination. The combined use of rapamycin and FTY720 showed additive and supra-additive antiproliferative effects on pancreatic carcinoma cell lines. The susceptibility of pancreatic carcinoma cells to rapamycin was significantly enhanced when combined with FTY720.

WITH THE DEVELOPMENT of immunosuppressive therapy, especially in the cyclosporine or tacrolimus era, many recipients have achieved long-term survivals after organ transplantation. But due to the lifelong immunosuppressive treatment and the resultant modification of the immune system, malignant tumors have become an increasingly important issue in the long-term follow-up of transplant recipients. After 10 years, up to 20% of graft recipients develop malignant tumors, reaching up to 40% after 20 years. Thus, malignancies contribute substantially to the morbidity and mortality of organ transplant patients.[1]

Malignancies develop in three ways: de novo occurrence in recipients; recurrent malignancy in recipients; or transmission of malignancy from the donors. Immunosuppressants such as cyclosporine and tacrolimus may contribute to the de novo development of cancer after organ transplantation.[2] Skin cancers are the most common malignant conditions in transplant recipients, followed by gynecological tumors and lymphomas.[3] Pancreatic carcinomas are

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This study is supported by a grant from the National Key Basic Research Program of China (No.2003CB515501).

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0041-1345/08/$–see front matter
doi:10.1016/j.transproceed.2008.03.150

frequent gastrointestinal tumors with an exceptionally bad prognosis. Their diagnosis is usually established at a progressive stage, necessitating adjuvant or neoadjuvant chemotherapy, although some patients received radical resection, the prognosis is disappointing, with a mean survival time of only 17 months.[4]

FTY720 and rapamycin both possess immunosuppressive as well as antiproliferative properties. Growth inhibition by both rapamycin and FTY720 has not only been demonstrated for lymphocytes, but also for vascular cells and tumor cells.[3,5,6]

FTY720, produced by modification of a metabolite from *Isaria sinclairii*, has been reported to induce apoptosis in lymphocytes; induce apoptosis; and prevent tumor growth and metastasis of several types of cancers, including breast, bladder, and liver cells.[7–9] Our previous study first demonstrated that FTY720 inhibited the growth, invasion, and metastasis of pancreatic carcinoma cell lines.[10]

Rapamycin targets the mammalian target of rapamycin (mTOR), leading to cell cycle arrest and inducing cell apoptosis. Its antitumor activity was recently demonstrated in various tumors, including pancreatic carcinoma. Additionally, a recent study demonstrated supra-additive antitumor effects when mycophenolate mofetil was combined with everolimus, a derivative of rapamycin.[2]

In the study presented here, we tested the in vitro antiproliferative capacity of rapamycin and FTY720 on pancreatic tumor cell lines Panc-1 and AsPc-1 using an MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide] (Sigma, St. Louis, MØ, USA) assay.

MATERIALS AND METHODS

Cell Lines

The pancreatic carcinoma cell lines of Panc-1 and AsPc-1 (Shanghai Institute of Cell Biology, Shanghai, China) were used as the in vitro model. The Panc-1 cells were routinely cultured in Dulbecco's modified Eagle's medium (HyClone, Utah, USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Kansas, USA), 100 U/mL penicillin G and 100 U/mL streptomycin. The AsPc-1 cells were cultured in RPMI-1640 (HyClone) containing 10% of FBS, 100 U/mL penicillin G and 100 U/mL streptomycin. These two cell lines were cultured in 5% CO_2 at 37°C. Subconfluent monolayer cells of Panc-1 or AsPc-1 were detached from the culture dishes by trypsin treatment, then centrifuged at 90*g* for 5 minutes and resuspended in the fresh media. Cells were seeded at a density of 1.0×10^4 cells per well in a 96-well flat bottom plates with a final volume of 200 μL for cell proliferation assays.

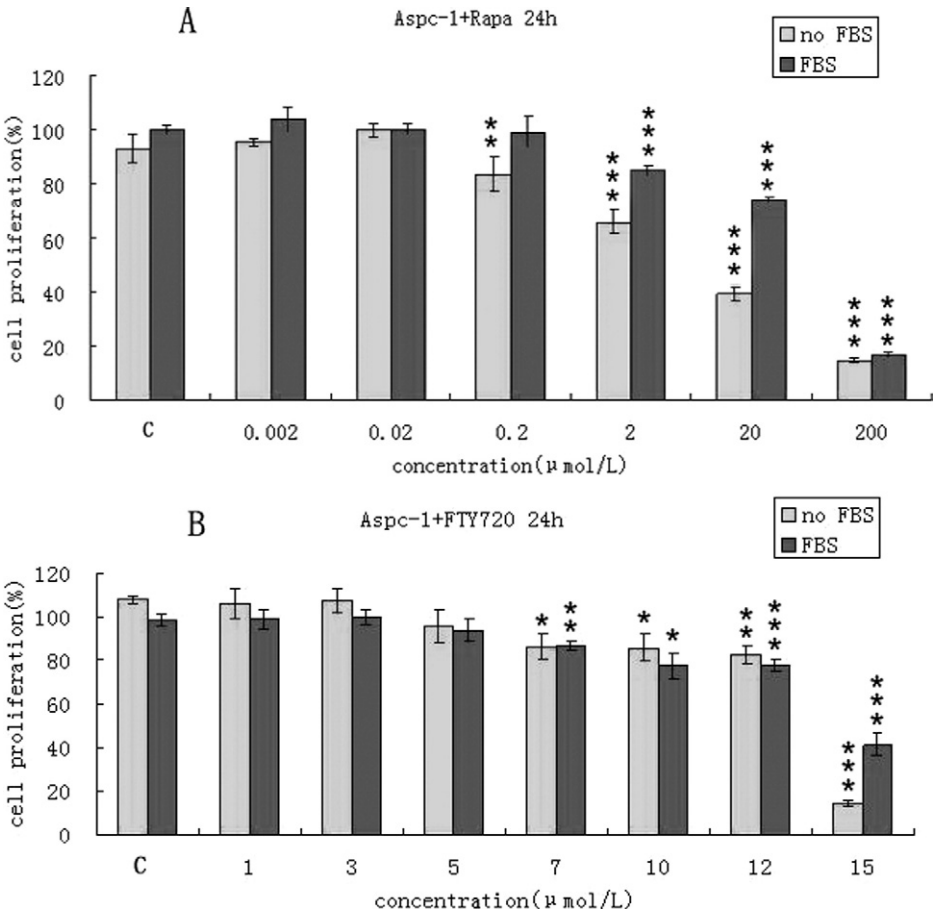

Fig 1. The proliferation of AsPc-1 cells could be dose-dependently inhibited after treatment by rapamycin alone (A) or FTY720 alone (B) for 24h with or without serum stimulation. The bivariate correlation test: r = −0.822, *P* = .000 for rapamycin with FBS; r = −0.863, *P* = .000 for rapamycin without FBS; r = −0.883, *P* = .000 for FTY720 with FBS; r = −0.889, *P* = .000 for FTY720 without FBS. The one-way ANOVA test: **P* < .05; ***P* < .01; ****P* < .001.

Reagents

The FTY720 was purchased from Cayman Chemical Company (Michigan, USA), the rapamycin, from BioSource International Inc (Camarillo, USA). Both rapamycin and FTY720 were dissolved in dimethylsulfoxide (DMSO). For monotreatment experiments, cells were treated with rapamycin in concentrations ranging from 0.002 μmol/L to 200 μmol/L or with FTY720 ranging from 1 μmol/L to 15 μmol/L either with or without FBS stimulation. For coincubation experiments, the FBS-containing culture mediums used two concentrations of rapamycin (2 μmol/L and 20 μmol/L) combined with seven concentrations of FTY720 (1 μmol/L, 3 μmol/L, 5 μmol/L, 7 μmol/L, 10 μmol/L, 12 μmol/L, and 15 μmol/L); or two concentrations of FTY720 (7 μmol/L and 10 μmol/L) with five concentrations of rapamycin (0.002 μmol/L, 0.02 μmol/L, 0.2 μmol/L, 2 μmol/L, and 20 μmol/L). Cells in culture medium containing DMSO vehicle were regarded as the control group.

Cell Proliferation

The antiproliferative capacity of the treatments was assessed by the MTT assay. The pancreatic carcinoma cell lines were treated with rapamycin or FTY720 at indicated concentrations for 24 hours, then the incubation medium was removed and 200 μL of fresh medium containing MTT (2.5 mg dissolved in 50 μL of DMSO) added to each well. After incubation for 4 hours at 37°C, the culture containing MTT was removed, 200 μL of DMSO was added to each well, and viable cells detected by measuring absorbance at 490 nm using MRX II absorbance reader (DYNEX Technologies, Chantilly, Virginia, USA). The cell viability was expressed as the percentage of absorbance in cells with FTY720 or rapamycin treatment versus the control group.

Statistical Analysis

All studies were performed three to six times, with results expressed as mean values ± standard deviations. A one-way ANOVA was used to compare the difference between the control group and each treated group; a bivariate correlation test was used to analyze the correlation between reagent concentrations and cell proliferation levels. All statistical analysis was performed with SPSS11.5 software with $P < .05$ considered to be significant.

RESULTS

For AsPc-1 cells, rapamycin and FTY720 showed dose-dependent inhibition of MTT incorporation with or without FBS stimulation (Fig 1). For rapamycin monotreatment under FBS stimulation, the inhibition levels by 2 μmol/L and 20 μmol/L rapamycin were about 15% and 30%, respectively. Stronger inhibition was observed without FBS stimulation, namely 30% and 60%, respectively (Fig 1A).

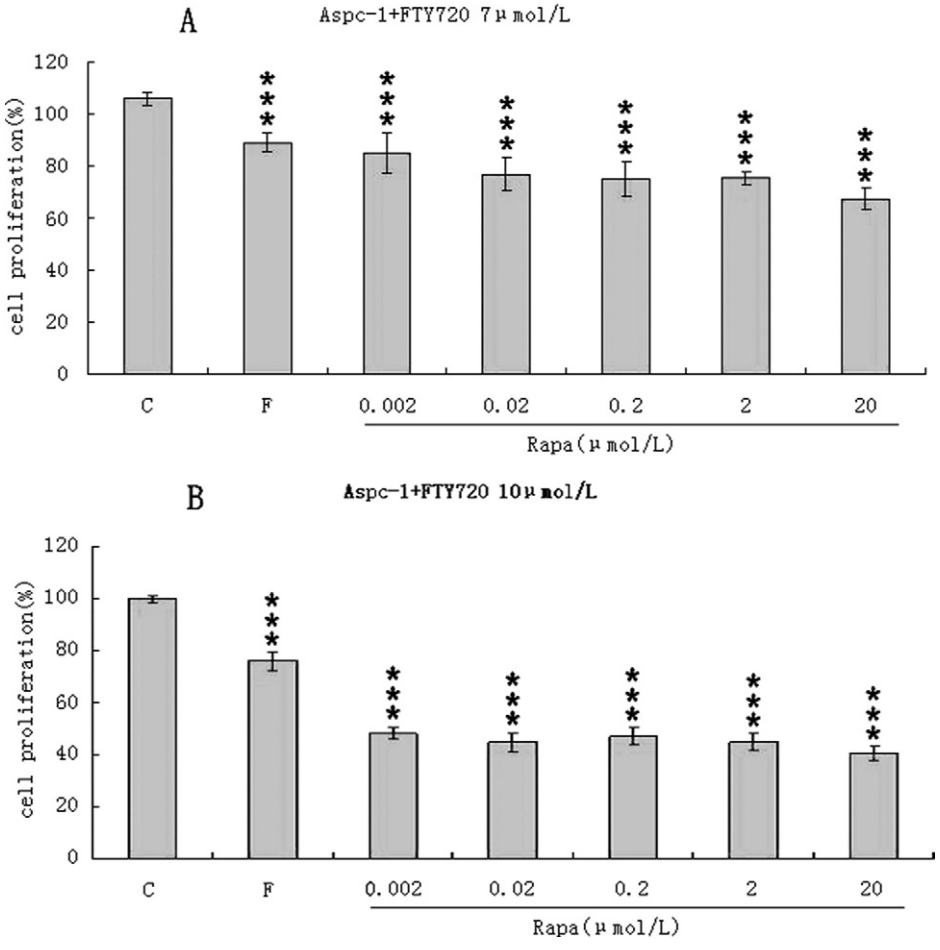

Fig 2. For coincubation experiments in AsPc-1, 7μM **(A)** and 10μM **(B)** FTY720 were combined with five indicated concentrations of rapamycin. Additive and supra-additive anti-proliferation effects were observed, the susceptibility of AsPc-1 to rapamycin was significantly enhanced when combined with FTY720. The one-way ANOVA test: ***$P < .001$.

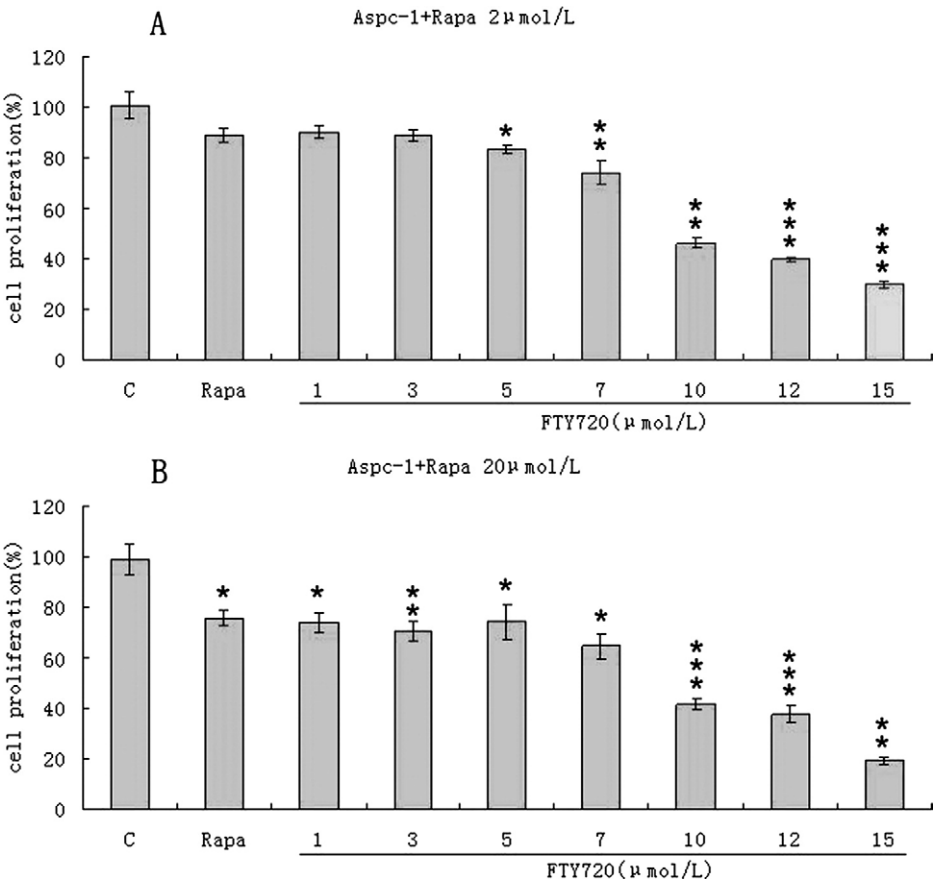

Fig 3. For coincubation experiments in AsPc-1, 2μM **(A)** and 20μM **(B)** rapamycin were combined with seven indicated concentrations of FTY720. Additive and supra-additive anti-proliferation effects were observed. The one-way ANOVA test: $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

For FTY720 monotreatment, the inhibition level of 7 μmol/L and 10 μmol/L FTY720 with FBS stimulation were about 15% and 20%, respectively; and were both about 15% without FBS stimulation (Fig 1B).

For coincubation experiments in AsPc-1 cells, 7 μmol/L and 10 μmol/L FTY720 were combined with five concentrations of rapamycin (Fig 2), and 2 μmol/L and 20 μmol/L rapamycin with seven concentrations of FTY720 (Fig 3). Interestingly, additive and supra-additive antiproliferative effects were observed; an effective combination was 10 μmol/L FTY720 with 0.002 μmol/L rapamycin, yielding more than 50% inhibition level (Fig 2B), which was greater than the levels of 20 μmol/L rapamycin and 12 μmol/L FTY720 used in monotreatment. Importantly, great susceptibility to rapamycin was observed when combined with 10 μmol/L FTY720, allowing the rapamycin concentration to be more than 100 times reduced.

For Panc-1 cells, rapamycin and FTY720 also produces dose-dependent inhibition of MTT incorporation with or without FBS stimulation (Fig 4). For rapamycin monotreatment in the condition of FBS stimulation, inhibition levels of 2 μmol/L and 20 μmol/L rapamycin were about 10% and 20%, respectively; stronger inhibition was observed without FBS stimulation, namely, 15% and 30%, respectively (Fig 4A). In monotreatment, the inhibition levels of 7 μmol/L and 10 μmol/L FTY720 with FBS stimulation were only about 6% and 10%, respectively; and 5% and 15%, respectively, without FBS stimulation (Fig 4B).

Similar to AsPc-1 cells, 2 μmol/L and 20 μmol/L rapamycin were combined with seven concentrations of FTY720 (Fig 5), and 7 μmol/L, and 10 μmol/L, FTY720 were combined with five concentrations of rapamycin (Fig 6). Additive and supra-additive antiproliferative effects were observed with Panc-1 cells. The effective combinations were 10 μmol/L FTY720 plus 0.002 μmol/L rapamycin and 10 μmol/L FTY720 plus 20 μmol/L rapamycin, the corresponding inhibition levels reached about 40% and 60%, respectively (Fig 6), more than the monotreatment effects of 20 μmol/L rapamycin and 12 μmol/L FTY720. The susceptibility of Panc-1 to rapamycin was also significantly enhanced more than 10-fold when combined with 10 μmol/L FTY720.

DISCUSSION

In this study, rapamycin and FTY720 showed dose-dependent antiproliferative effects on Panc-1 and AsPc-1 pancreatic carcinoma cell lines in vitro with or without FBS stimulation. Importantly, supra-additive antiproliferative effects were observed with combined treatment of rapamycin and

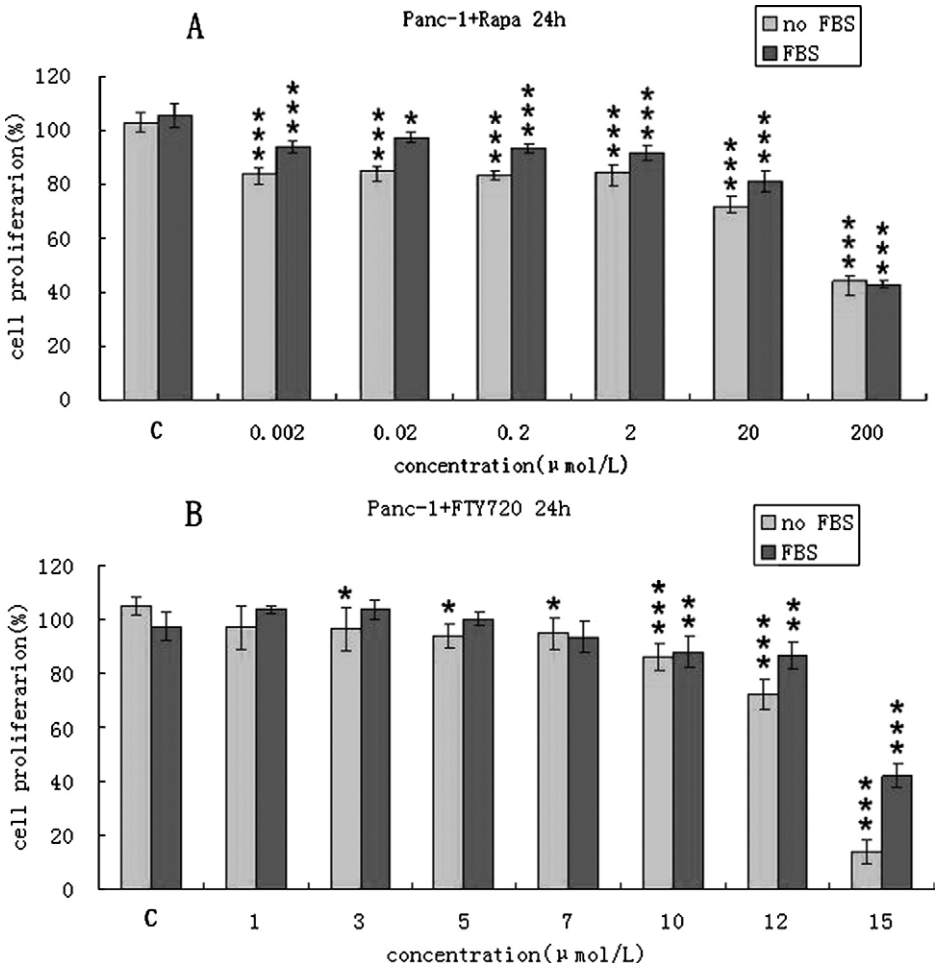

Fig 4. The proliferation of Panc-1 cells could be dose-dependently inhibited after treatment by rapamycin alone **(A)** or FTY720 alone **(B)** for 24h with or without serum stimulation. $P < .05$. The bivariate correlation test: $r = -0.878$, $P = .000$ for rapamycin with FBS; $r = -0.801$, $P = .000$ for rapamycin without FBS; $r = -0.785$, $P = .000$ for FTY720 with FBS; $r = -0.850$, $P = .000$ for FTY720 without FBS. The one-way ANOVA test: $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

FTY720. The susceptibility of pancreatic carcinoma cells to rapamycin was significantly enhanced when combined with FTY720, leading to a 10- to 100-fold reduction in rapamycin concentration.

Rapamycin has shown potential for application for cancer treatment.[11,12] It inhibits the proliferation of various tumor cell lines in vitro.[13] Human pancreatic tumors have been reported to be sensitive to CCI-779 (a rapamycin ester formulated for intravenous use), and mTOR signaling appears to be required for pancreatic cancer cell proliferation.[11] Antitumor efficacy of a rapamycin derivative has been shown in one animal model of pancreatic cancer.[14] Our study also demonstrated dose-dependent antiproliferative effects on pancreatic carcinoma cells.

Recently, some studies have reported FTY720 as a possible antitumor agent.[7–9] Three recent in vivo studies showed that FTY720 prevented tumor growth and metastases of breast, bladder, and liver xenografts in nude mice.[7,8] Our previous study confirmed that FTY720 suppressed the proliferation, organization, migration, and invasion of pancreatic carcinoma cells even at low concentrations. Additionally, FTY720 induced apoptosis of pancreatic carcinoma cells, possibly via down-regulation of Akt phosphorylation and Bcl-2 expression.[10] In this study FTY720 also was demonstrated to inhibit the proliferation of pancreatic carcinoma cells in a dose-dependent manner. The combined use of rapamycin and FTY720 showed additive and supra-additive antiproliferative effects on pancreatic carcinoma cells. Importantly, the use of FTY720 may significantly enhance the antiproliferative effects of rapamycin on pancreatic carcinoma cells.

A previous study recommended a whole-blood rapamycin therapeutic window of 5 to 15 ng/mL (5.5 nmol/L to 16.4 nmol/L) for patients at standard risk of rejection.[15] Additionally, it was reported that the FTY720 plasma peak concentration of 2.8 ng/mL (8.15 nmol/L) would not induce severe toxicity in renal transplantation recipients.[16] Thus, the concentration ranges of both rapamycin and FTY720 in this study were markedly higher than those observed in vivo, which may contribute to the observed difference. To date, the effects of the drug concentration ranges and combinations used in this experiment on normal epithelial or endothelial cell lines or in animal models remain uncertain. We performed studies focused on the effects of

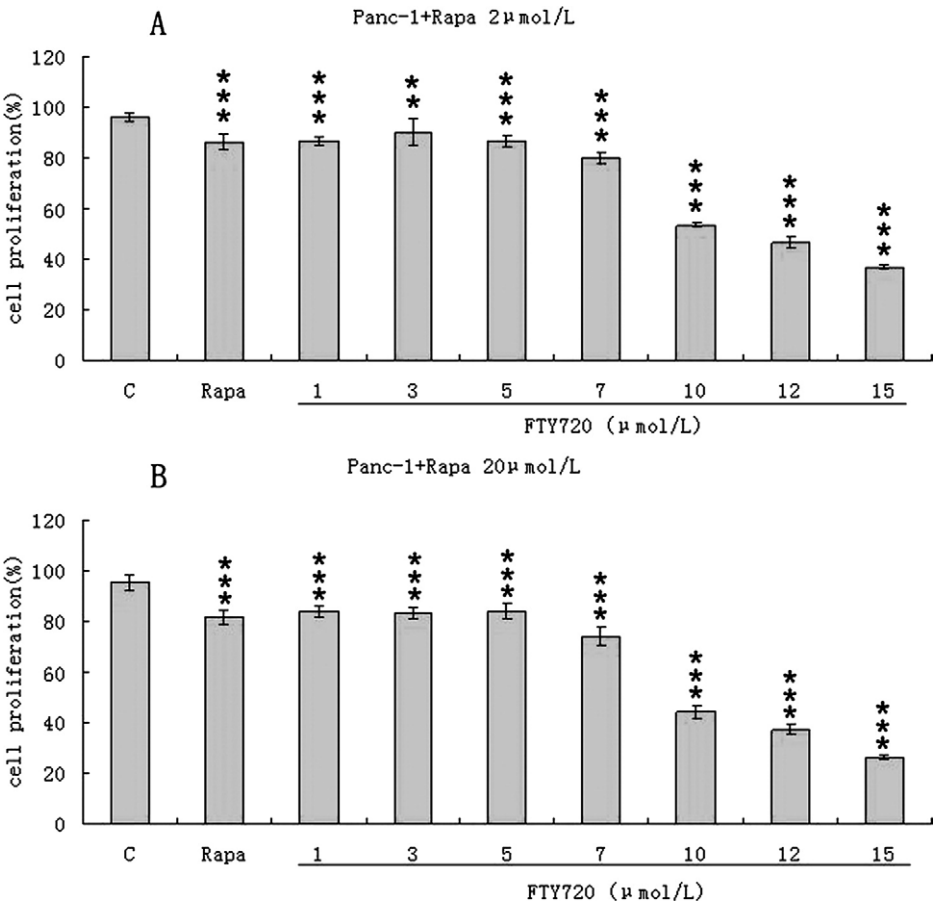

Fig 5. For coincubation experiments in Panc-1, 2μM **(A)** and 20μM **(B)** rapamycin were combined with seven indicated concentrations of FTY720. Additive and supra-additive anti-proliferation effects were observed. The one-way ANOVA test: $^{**}P < .01$; $^{***}P < .001$.

FTY720 in a nude mouse model of pancreatic cancer and found that FTY720 (10 mg/kg per day) did not cause severe toxicity in nude mice. In addition, the safe ranges and combinations of FTY720 and rapamycin in normal epithelial or endothelial cell lines, or animal models must be the focus of our next study.

In conclusion, the immunosuppressive drugs rapamycin and FTY720 show additional antitumor effects. We showed in vitro antiproliferative capacities in pancreatic cancer cell lines Panc-1 and AsPc-1, both when used as single substances and in combination. Additive and supra-additive antiproliferative effects were observed in combined treatment. The susceptibility of pancreatic carcinoma cells to rapamycin was significantly enhanced when combined with FTY720.

REFERENCES

1. Lutz J, Heemann U: Tumors after kidney transplantation. Curr Opin Urol 13:105, 2003

2. Stracke S, Ramudo L, Keller F, et al: Antiproliferative and overadditive effects of everolimus and mycophenolate mofetil in pancreas and lung cancer cells in vitro. Transplant Proc 38:766, 2006

3. Eisen HJ, Tuzcu EM, Dorent R, et al: RAD B253 Study Group: Rapamycin for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. N Engl J Med 349:847, 2003

4. Berlin JD, Catalano P, Thomas JP, et al: Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. J Clin Oncol 20:3270, 2002

5. Ho JW, Man K, Sun CK, et al: Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma. Mol Cancer Ther 4:1430, 2005

6. Boulay A, Zumstein-Mecker S, Stephan C, et al: Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. Cancer Res 64:252, 2004

7. Azuma H, Takahara S, Ichimaru N, et al: Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. Cancer Res 62:1410, 2002

8. Azuma H, Takahara S, Horie S, et al: Induction of apoptosis in human bladder cancer cells in vitro and in vivo caused by FTY720 treatment. J. Urol 169:2372, 2003

9. Sonoda Y, Yamamoto D, Sakura S, et al: FTY720, a novel immunosuppressive agent, induces apoptosis in human glioma cells. Biochem Biophys Res Commun 281:282, 2001

10. Shen Y, Cai M, Xia W, et al: FTY720, a synthetic compound from Isaria sinclairii. Cancer Lett 254:288, 2007

11. Hidalgo M, Rowinsky EK: The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene 19:6680, 2000

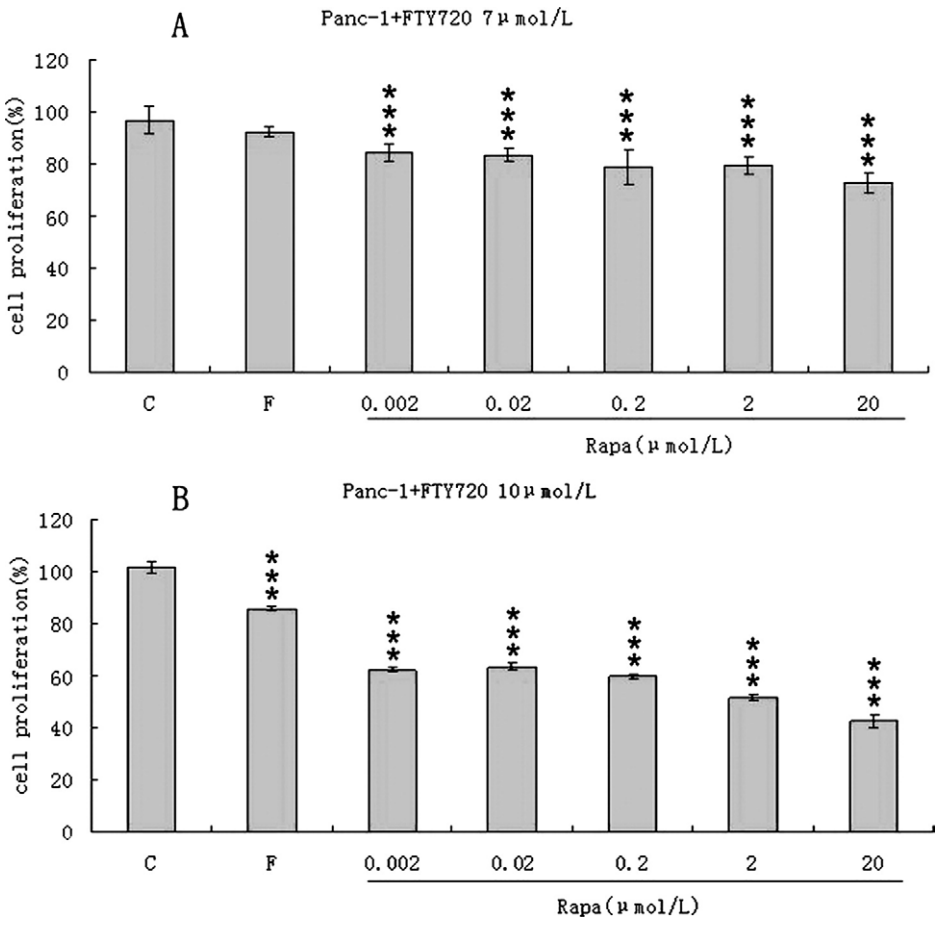

Fig 6. For coincubation experiments in Panc-1, 7μM **(A)** and 10μM **(B)** FTY720 were combined with five indicated concentrations of rapamycin. Additive and supra-additive anti-proliferation effects were observed. The one-way ANOVA test: ***$P < .001$.

12. Grewe M, Gansauge F, Schmid RM, et al: Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6K pathway in human pancreatic cancer cells. Cancer Res 59:3581, 1999

13. Huang S, Houghton PJ: Inhibitors of mammalian target of rapamycin as novel antitumor agents: from bench to clinic. Curr Opin Invest Drugs 3:295, 2002

14. Boulay A, Zumstein-Mecker S, Stephan C, et al: Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. Cancer Res 64:252, 2004

15. MacDonald A, Scarola J, Burke JT, et al: Clinical pharmacokinetics and therapeutic drug monitoring of sirolimus. Clin Ther 22(Suppl B):B101, 2000

16. Budde K, Schmouder RL, Brunkhorst R, et al: First human trial of FTY720, a novel immunomodulator, in stable renal transplant patients. J Am Soc Nephrol 13:1073, 2002