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Targeting the JNK signaling pathway potentiates the antiproliferative efficacy of Rapamycin in LS174T colon cancer cells.

THESE

préparée sous la direction du Professeur Nicolas Demartines MD
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par

Michael BENOIT

Médecin diplômé de la Confédération Suisse
Originaire de La Sagne et Les-Ponts-de-Martel, Neuchâtel, Suisse

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Co-Directeur de thèse	
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Directrice de l'Ecole doctorale	Madame le Professeur **Stephanie Clarke**

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Directrice de l'Ecole doctorale

Résumé

Les récentes thérapies anticancéreuses développées visent principalement à inhiber les protéines mutées et responsables de la croissance des cellules cancéreuses. Dans ce contexte, l'inhibition d'une protéine appelée mTOR est une stratégie prometteuse. En effet, mTOR régule la prolifération et la survie cellulaire et mTOR est fréquemment activé dans les cellules tumorales.

De nombreuses études ont démontré l'efficacité anti-tumorale d'inhibiteurs de mTOR telle que la rapamycine aussi bien dans des modèles expérimentaux que chez les patients souffrant de cancers. Ces études ont cependant également démontré que l'inhibition de mTOR induit l'activation d'autres protéines cellulaires qui vont induire la prolifération cellulaire et ainsi limiter l'effet anti-tumoral des inhibiteurs de mTOR. En particulier, la rapamycine induit l'activation de la voie de signalisation PI3K/Akt qui joue un rôle prépondérant dans la croissance cellulaire.

Dans ce travail, nous avons étudié l'effet de la rapamycine sur une protéine appelée JNK ainsi que le rôle de JNK sur les effets anti-tumoraux de la rapamycine. JNK est une protéine impliquée dans la survie et la prolifération cellulaire. Elle est activée notamment par la voie de signalisation PI3K/Akt. De ce fait, nous avons émis l'hypothèse que la rapamycine induirait l'activation de JNK, réduisant ainsi l'efficacité anti-tumorale de la rapamycine. En utilisant une lignée cellulaire tumorale (LS174T) dérivée du cancer colorectal, nous avons observé que la rapamycine induisait l'activation de JNK. Nous avons également observé que l'inhibition de JNK par le SP600125, un inhibiteur chimique de JNK, ou par la surexpression d'un dominant négatif de JNK dans les cellules LS174T potentialisait l'effet anti-tumoral de la rapamycine *in vitro* ainsi que dans un modèle murin de xénogreffe tumorale *in vivo*.

En conclusion, nous avons observé que l'activation de JNK induite par la rapamycine entraine une réduction de l'effet anti-tumoral de cette dernière. Nous proposons ainsi que l'inhibition simultanée de JNK et de mTOR représente une nouvelle option thérapeutique en oncologie qu'il conviendra de confirmer dans d'autres modèles expérimentaux avant d'être testée dans des études cliniques.

Targeting the JNK Signaling Pathway Potentiates the Antiproliferative Efficacy of Rapamycin in LS174T Colon Cancer Cells

Michael Benoit, M.D., Anne Dormond-Meuwly, M.D., Nicolas Demartines, M.D., and Olivier Dormond, M.D., Ph.D.[1]

Department of Visceral Surgery, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Switzerland

Background. **Targeting the mTOR signaling pathway with rapamycin in cancer therapy has been less successful than expected due in part to the removal of a negative feedback loop resulting in the overactivation of the PI3K/Akt signaling pathway. As the c-Jun N-terminal kinase (JNK) signaling pathway has been found to be a functional target of PI3K, we investigate the role of JNK in the anticancer efficacy of rapamycin.**

Materials and Methods. **The colon cancer cell line LS174T was treated with rapamycin and JNK phosphorylation was analyzed by Western Blot. Overexpression of a constitutively negative mutant of JNK in LS174T cells or treatment of LS174T cells with the JNK inhibitor SP600125 were used to determine the role of JNK in rapamycin-mediated tumor growth inhibition.**

Results. **Treatment of LS174T cells with rapamycin resulted in the phosphorylation of JNK as observed by Western Blot. The expression of a negative mutant of JNK in LS174T cells or treatment of LS174T cells with SP600125 enhanced the antiproliferative effects of rapamycin. In addition, *in vivo*, the antitumor activity of rapamycin was potentiated on LS174T tumor xenografts that expressed the dominant negative mutant of JNK.**

Conclusions. **Taken together, these results show that rapamycin-induced JNK phosphorylation and activation reduces the antitumor efficacy of rapamycin in LS174T cells.** © 2011 Elsevier Inc. All rights reserved.

Key Words: **mTOR; rapamycin; JNK; proliferation; tumor; signaling.**

[1] To whom correspondence and reprint requests should be addressed at Department of Visceral Surgery, Pavillon 3, CHUV, Avenue de Beaumont, 1011 Lausanne, Switzerland. E-mail: olivier.dormond@chuv.ch.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a highly conserved ser/thr kinase that plays a key role in cell growth, proliferation and survival. mTOR regulates cell growth in response to growth factors, nutrients and energy [1]. mTOR is present in two functionally distinct complexes, mTORC1 and mTORC2. mTORC1 consists of mTOR, raptor, deptor, PRAS40, and mLST8. mTORC2 is composed of mTOR, rictor, sin1, mLST8, deptor, and protor [2]. While acute exposure to rapamycin blocks mTORC1, it does not affect mTORC2 [3]. Despite several experimental evidences in different animal models that the inhibition of mTORC1 by rapamycin and its analogs blocks tumor progression, its benefits in clinical trials have been less successful than expected [4, 5]. Part of it might be explained by the observation that the inhibition of mTORC1 results in the activation of the PI3K/Akt signaling pathway, which induces signals that sustain proliferation and survival and, thus, counteract the anti-cancer efficacy of rapamycin [6].

Jun N-terminal kinases (JNKs), also known as stress-activated MAP kinases (SAPK), belong to the family of MAP kinases that regulate cell differentiation, proliferation, and apoptosis. The JNK protein kinases are encoded by three genes. While JNK1 and JNK 2 are ubiquitously expressed, JNK3 expression is limited to brain, heart, and testis [7, 8]. Although several reports have unravelled the critical role of JNK in inducing apoptosis, several evidences have also demonstrated a role of JNK in cancer development by enhancing cell proliferation and survival [7–10]. Indeed, the requirement of JNK in the development of various cancers including colon, ovarian as well as prostate cancer has been suggested [11–14]. Interestingly,

constitutively active alleles of PI3K or activation of PI3K following PTEN loss activate JNK resulting in cell transformation [15, 16].

Several studies have demonstrated that rapamycin induces the activation of proliferative and prosurvival effectors such as PI3K, which limits its anticancer efficacy. Since JNK is a functional target of PI3K, we want here to determine the effect of rapamycin on JNK signaling pathway and to explore the anticancer efficacy of the simultaneous inhibition of mTOR and JNK.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Reagents

The LS174T colon cancer cell line was purchased from the American Type Culture Collection (ATCC, Molsheim, France) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Rapamycin was obtained from LC Laboratories (Woburn, MA). Antibodies directed against phospho-Akt (S473), Akt, phospho JNK (Thr 183/Tyr 185), JNK, phospho-S6 ribosomal protein (Ser 235/236), and S6 ribosomal protein were from Cell Signaling Technology (Danvers, MA). Antibody to FLAG was obtained from Sigma (Sigma-Aldrich, Buchs, Switzerland). The dominant negative mutant JNK1 (plasmid 13846) and pcDNA3 (plasmid 10792) were obtained from Addgene (Cambridge, MA) [17].

Cell Transfection

LS174T cells were transfected with pcDNA3 or a flag tagged dominant negative mutant of JNK1 using the Effectene transfection reagent (Qiagen, Basel, Switzerland) and following the manufacturer's instructions. Stable transfectants were selected for resistance to G418 (500 μg/mL) for 2 wk and further expanded in the presence of G418 (100 μg/mL).

Western Blot Analysis

LS174T cells were treated with rapamycin (10 ng/mL) for 30 min, 1 or 24 h or dimethylsulfoxide (DMSO) as a control. Cells were lysed in RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 1 mM sodium orthovanadate. Protein concentrations were determined using BCA Assay (Pierce, Rockford, IL). Twenty μg of proteins were separated on 4%–12% polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Zug, Switzerland). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) and immunoblotted with primary antibodies followed by infrared secondary antibodies. Bands from immunoreactive proteins were visualized by an Odyssey infrared imaging system.

MTS Proliferation Assay

MTS proliferation studies were carried out using the CellTiter 96 Aqueous One Solution (Promega, Dübendorf, Switzerland). LS174T cells were plated on 96 well plates and cultured in DMEM 10% FBS. Twenty-four hours later cells were treated with rapamycin (10 ng/mL), SP600125 (10 μM) a combination of both drugs and DMSO. Forty-eight hours later, proliferation was monitored with the colorimetric assay by measuring the absorbance at 490 nm. Results are expressed as the mean absorbance ± SD of one representative experiment carried out in triplicate.

Cell Proliferation Studies

LS174T cells were plated in 6-well culture plates at a density of 200,000 cells/well and cultured in DMEM supplemented with 10% fetal calf serum. Twelve hours later, cells were treated with rapamycin (10 ng/mL) SP600125 (10 μM) a combination of both drugs or DMSO as a control and incubated for 3, 5 or 7 days. Cells were subsequently collected and trypan-blue negative cells were counted by light microscopy using a Neubauer hemocytometer.

Apoptosis Assay

LS174T cells were plated on 96 well plates and cultured in DMEM 10% FBS. Twenty-four hours later, cells were treated with rapamycin (10 ng/mL), SP600125 (10 μM) a combination of both drugs and DMSO for 48 h. Subsequently, cells were harvested and apoptosis was monitored using the Cell Detection ELISA Plus (Roche Diagnostics, Rotkreuz, Switzerland) following the manufacturer's instructions.

Tumor Xenografts

Animal experiments were in accordance with the Swiss Federal Animal Regulations and approved by the local veterinary office. Female nude mice aged 8 wk were purchased from Charles River (Charles River Laboratories, St. Germain sur l'Arbresle, France). One million LS174T cells or LS174T cells stably transfected with pcDNA3 or a dominant negative mutant of JNK were injected subcutaneously (s.c.) into the flank of nude mice. Once the tumor xenografts reached 100 mm³, mice were randomized into different groups (n = 5/group) and treated with vehicle (ethanol) or rapamycin (1.5 mg/kg/d) for 20 d. All mice were sacrificed after 20 d of treatment. Tumor volumes were measured using callipers every day and calculated with the formula V = π/(6a²b) where a is the short axis and b the long axis of the tumor.

Statistical Analysis

Data were analyzed by Student's t-test or one way ANOVA. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Rapamycin Increases the Phosphorylation of JNK in LS174T Cells

To determine whether rapamycin induces the activation of JNK, LS174T colon cancer cells were treated with increasing concentrations of rapamycin and JNK phosphorylation was determined by Western Blot analysis as a read-out of JNK activity. We found that treatment of LS174T cells with 10 ng/mL of rapamycin increased JNK phosphorylation (Fig. 1A). At this concentration, rapamycin also totally blocked mTORC1 activity as assessed by the dephosphorylation of S6 ribosomal protein, a downstream effector of mTORC1. A lower dose of rapamycin (1 ng/mL) had no effect on JNK phosphorylation and did not block mTORC1 (Fig. 1A). The effect of rapamycin on JNK phosphorylation was apparent after 30 min and persisted for 24 h (Fig.1B). In addition, treatment of LS174T cells with rapamycin increased Akt phosphorylation, consistent

A
Rapamycin (ng/ml)   -   1   10   100

pJNK
JNK
pS6
S6

B
Rapamycin (h)   -   0.5   1   24

pJNK
JNK
pS6
S6
pAkt
Akt
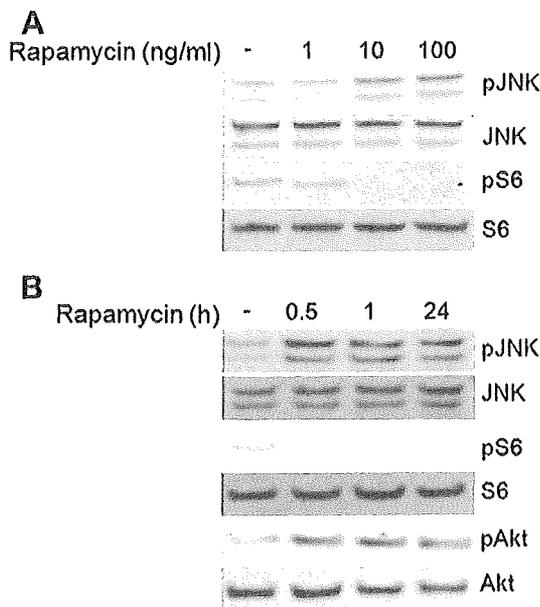
FIG. 1. Rapamycin increases JNK phosphorylation in LS174T cells. (A) LS174T colon cancer cells were treated with increasing concentrations of rapamycin for 1 h or DMSO as a control. Cell lysates were analyzed by Western Blot for phospho-JNK (Thr 183/Tyr 185), JNK, phospho-S6 ribosomal protein (Ser 235/236), and S6 ribosomal protein. (B) LS174T colon cancer cells were treated with rapamycin (10 ng/mL) or DMSO as a control for the indicated times. Cell lysates were analyzed by Western Blot for phospho-JNK (Thr 183/Tyr 185), JNK, phospho-S6 ribosomal protein (Ser 235/236), S6 ribosomal protein, phospho Akt (Ser 473), and Akt. The illustrated blots are representative of three similar experiments.

with the removal of a negative feedback loop whereby the inhibition of mTORC1 and its downstream substrate S6K1 results in the activation of the PI3K/Akt signaling pathway (Fig.1B) [6]. Taken together, these results show that rapamycin induces the phosphorylation of JNK.

SP600125 Potentiates the Antiproliferative Efficacy of Rapamycin

We next investigated whether rapamycin-mediated JNK activation reduces the antiproliferative efficacy of rapamycin in LS174T cells. To test this, LS174T cells were treated with the JNK inhibitor SP600125, rapamycin, a combination of both or left untreated and cell proliferation was analyzed by MTS assay after 48 h of treatment. In preliminary experiments, we observed that 10 μM of SP600125 was needed to block JNK activity in LS174T cells (data not shown). Both rapamycin and SP600125 reduced cell proliferation when used as a single agent (Fig. 2A). However the combination of both had an additive effect that was statistically significant compared with the single treatments. Consistent with this finding, we also found that rapamycin or SP600125 reduced LS174T cell number after 3, 6, and

A

B

C
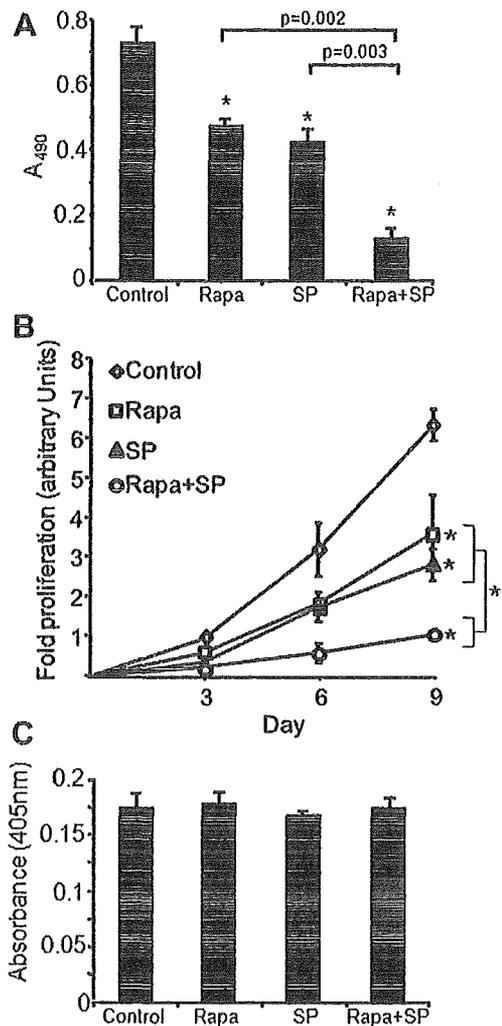
FIG. 2. The inhibition of JNK with SP600125 increases the antiproliferative effect of rapamycin in LS174T cells. (A) LS174T cells were treated with rapamycin (rapa, 10 ng/mL) or SP600125 (SP, 10 μM) alone or a combination of both drugs for 48 h. Proliferation was monitored by an MTS assay. Results are expressed as the mean absorbance at 490 nm $\pm$ SD of one representative experiment carried out in triplicate. P values were calculated by using a Student's t-test. *$P < 0.005$ compared with control. (B) LS174T cells were processed as under (A). Subsequently, cells were collected after 3, 6, or 9 days of treatment and trypan blue negative cells were counted by light microscopy using a Neubauer hemocytometer. Mean cell counts of three independent experiments were expressed in arbitrary units $\pm$ SD. *$P < 0.05$ compared with control or as specified by brackets (Student's t-test). (C) LS174T cell were processed as under (A). Cells were harvested and apoptosis was determined by DNA fragmentation. Results are represented as the mean absorbance at 405 nm $\pm$ SD ($n = 3$).

9 d of treatment (Fig. 2B). This reduction was also more significant when rapamycin was used in combination with SP600125 compared to either drug alone. Finally the effect of rapamycin and SP600125 on LS174T cell apoptosis was monitored by quantifying DNA fragmentation. Rapamycin and SP600125 either as single agent or in combination did not induce cell apoptosis (Fig. 2C).

The Antiproliferative Efficacy of Rapamycin is Enhanced by the Expression of a Dominant Negative Mutant of JNK in LS174T Cells
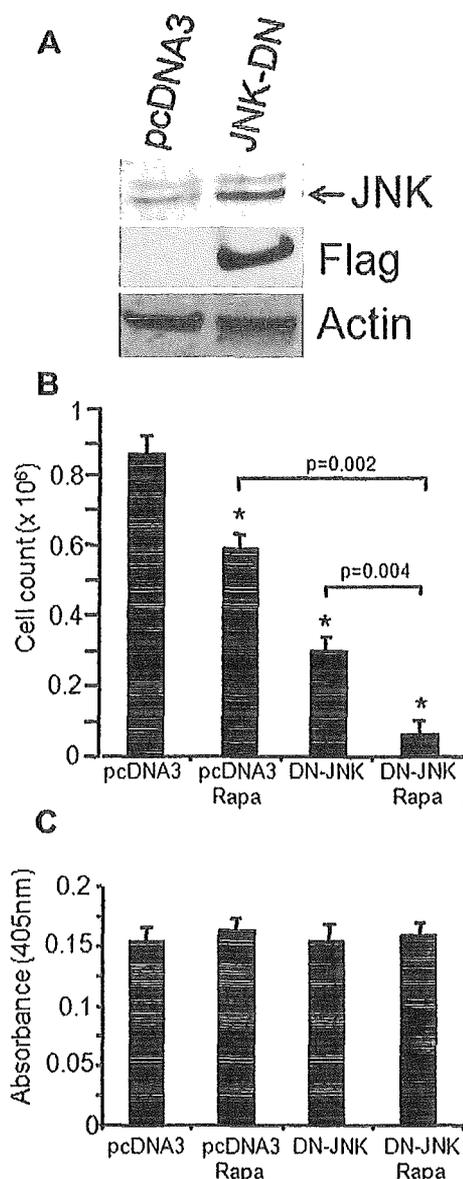
To further explore the role of JNK in the antiproliferative efficacy of rapamycin, we stably transfected LS174T cells with a dominant negative mutant of JNK1 (DN-JNK) or an empty vector as a control (pcDNA3) [17]. The expression of the dominant negative mutant of JNK1 was confirmed by Western blot analysis using an anti-flag as well as an anti-JNK antibody (Fig. 3A). We found that the expression of DN-JNK in LS174T cells decreased cell proliferation compared with pcDNA3 transfected cells. Furthermore, the antiproliferative efficacy of rapamycin was greater on DN-JNK transfected cells compared with cells transfected with pcDNA3 (Fig. 3B). Finally, similar to what we found with SP600125 (Fig. 2C), the expression of DN-JNK did not induce cell apoptosis even in the presence of rapamycin (Fig. 3C).

The Anticancer Efficacy of Rapamycin is Enhanced in LS174T Tumor Xenografts that Express a Dominant Negative Mutant of JNK

We next evaluated the relevance of rapamycin-mediated JNK activation on the antitumor efficacy of rapamycin *in vivo*. We first determined whether rapamycin increased JNK phosphorylation in LS174T tumor xenografts. LS174T cells were injected subcutaneously into immunodeficient mice that were subsequently treated with rapamycin or not. Western Blot analysis of the tumor lysates revealed that rapamycin increased the phosphorylation of JNK (Fig. 4A). We further analyzed whether rapamycin-mediated JNK activation counteracts the growth inhibitory effects of rapamycin. To test this, LS174T cells that were stably transected with DN-JNK or pcDNA3 were injected into nude mice. We observed that the growth of the xenografts expressing the dominant negative mutant of JNK was reduced. Moreover, we found that the antitumor efficacy of rapamycin was potentiated in tumor xenografts expressing the dominant negative mutant of JNK (Fig. 4B). Taken together, these results suggest that rapamycin-mediated JNK activation reduces the antitumor efficacy of rapamycin.

DISCUSSION

In this report, we have found that the treatment of LS174T cells with rapamycin induced the phosphorylation of JNK and that the expression of a dominant negative mutant of JNK or the exposure of LS174T cells to SP600125 potentiated rapamycin growth inhibitory effects.

FIG. 3. The expression of a dominant negative mutant of JNK in LS174T cells enhances the antiproliferative efficacy of rapamycin. (A) LS174T cells were stably transfected with an empty vector (pcDNA3) or a dominant negative mutant of JNK (DN-JNK). Cells were lysed and analyzed by Western blot for DN-JNK using an anti-flag and an anti-JNK antibody. Actin was used as a loading control. (B) LS174T cells were stably transfected with a dominant negative mutant of JNK or pcDNA3 as a control were treated with rapamycin (Rapa, 10 ng/mL) for 72 h. Cells were subsequently collected and trypan blue negative cells were counted by light microscopy using a Neubauer hemocytometer. Results are illustrated as the mean cell count of three representative experiments ± SD. *P < 0.005 compared with control (Student's t-test). (C) LS174T were processed as under (B). Apoptosis was determined by DNA fragmentation and are expressed as the mean absorbance at 405 nm ± SD (n = 3).

Experimental studies and clinical trials have shown that the blockade of one molecular target in cancer therapy results in limited benefits [18, 19]. Therefore, optimal therapeutic approaches involve the inhibition of multiple molecular targets. Consistent with this
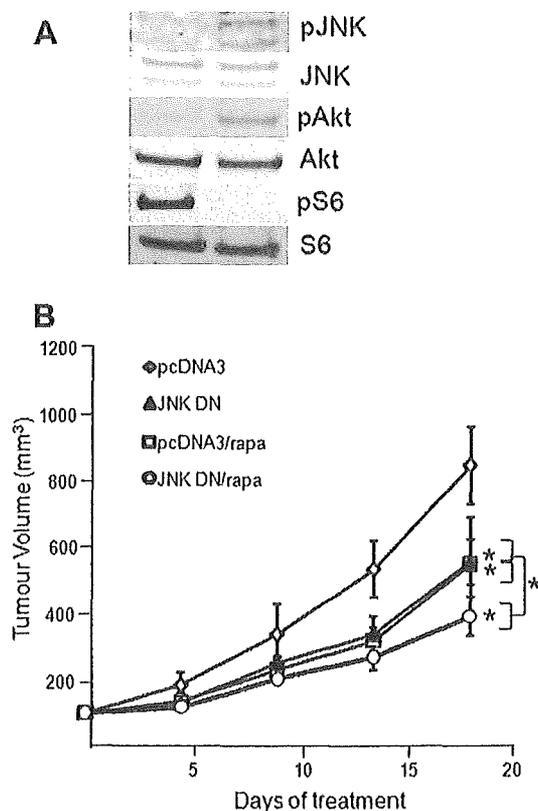
FIG. 4. The expression of a dominant negative mutant of JNK in LS174T xenografts potentiates the anticancer efficacy of rapamycin. (A) LS174T cells were injected into nude mice. Once the tumor reached 100 mm³, mice were treated with vehicle or rapamycin (1.5 mg/kg/d). Following 20-da of treatment, tumors were harvested and analyzed by Western Blot for pJNK, JNK, phospho-S6 ribosomal protein, and S6 ribosomal protein. (B) LS174T were stably transfected with a plasmid encoding a dominant negative mutant of JNK or with pcDNA3. Cells were subsequently injected into the flank of nude mice, and mice were either treated with vehicle or with rapamycin (1.5 mg/kg/d, intraperitoneally) for 20 d (n = 5/group). Points: average value of tumor volume. Bars: SD. *P < 0.05 compared with pcDNA3 or as specified by brackets (one-way ANOVA).

paradigm, the use of rapamycin as single agent in various clinical studies was associated with sporadic responses and combined therapy has potentiated the anticancer efficacy of rapamycin [4, 5]. Indeed, combining treatment that target the epidermal growth factor receptor, FOXO1, MEK, or DNA methyltransferase potentiated the anticancer efficacy of rapamycin [20–23]. We further show here that combining JNK and mTOR inhibitors have additive antiproliferative effect on LS174T cells. Indeed, simultaneous inhibition of JNK and mTOR in LS174T resulted in reduced tumor growth both in vitro and in vivo that was superior to single inhibition (Fig. 2B, 3B, and 4B). Therefore, combining JNK and mTOR inhibitors may provide a novel strategy in cancer therapy.

The strategy to combine rapamycin with other signaling pathway inhibitors relies also on a biological rationale. In fact, the inhibition of mTORC1 by rapamycin

stops a negative feedback loop whereby S6K1 signals back to PI3K [6]. This results in the activation of proliferative and prosurvival signals, including the PI3K/Akt and the RAS/RAF/MEK/Erk signaling pathways, which counteract the anticancer efficacy of rapamycin. Similarly, we have found in LS174T cells that rapamycin induces the activation of Akt, suggesting the removal of a negative feedback loop. Our data further show that rapamycin leads to the phosphorylation and activation of JNK signaling pathway. Consistent with this finding, it has been reported that rapamycin activates JNK in a human glioblastoma cell line that expresses a constitutively active mutant of the epidermal growth factor receptor [24]. Furthermore, rapamycin also increased JNK phosphorylation in MIN6 cells and Wistar rat pancreatic ilets [25]. Finally rapamycin was also shown to induce a transient activation of JNK in cells expressing wild-type p53 and a sustained activation in cells expressing mutant p53 [26].

One mechanism regulating rapamycin-mediated JNK activation involves the apoptosis signal-regulating kinase 1 (ASK1). Under serum-free conditions, rapamycin activates ASK1 by inhibiting protein phosphatase 5 [27]. In turn, the activation of ASK1 leads to the up-regulation of JNK signaling pathway. Interestingly, ASK1 mediated JNK activation depends on the expression of 4E-BP1, a downstream effector of mTOR, and not of S6K1. This suggests that rapamycin activates JNK independently of the S6K1/PI3K feedback loop. In this study, we have not found any change in the phosphorylation status of ASK-1 following rapamycin treatment, suggesting that another mechanism regulates rapamycin-mediated JNK activation in LS174T cells (data not shown). Furthermore, rapamycin-mediated ASK1/JNK activation induces cell apoptosis which was not observed in LS174T cells.

Since rapamycin increases PI3K activity in LS174T cells and since JNK is a functional downstream target of PI3K, we initially hypothesized that rapamycin-mediated JNK activation was PI3K-dependent [16]. However, the use of LY294002, a PI3K inhibitor, failed to block rapamycin-mediated JNK phosphorylation (data not shown). Therefore, further studies are needed to describe the mechanism by which rapamycin-induces JNK phosphorylation in LS174T cells.

Our study shows that rapamycin-induced JNK activation was detrimental to the anticancer efficacy of rapamycin, providing an experimental evidence to combine JNK and mTOR inhibitors. However, one may speculate that blocking JNK would potentiate the effectiveness of rapamycin regardless of whether rapamycin induces JNK activation or not. In fact, we found that JNK and mTOR inhibitors had also additive antiproliferative effects in HT29 cells in which rapamycin did not induce JNK activation (data not shown).

Therefore, the strategy of combining mTOR and JNK inhibitors may not be restricted to cancer cells in which rapamycin activates JNK. Thus, to apply these findings to a clinical setting, it will be important to identify biomarkers for cancer cells that are likely to respond to combined mTOR and JNK inhibition rather than identify cells in which rapamycin induces JNK activation.

Emerging evidences show that JNK is implicated in oncogenic transformation, particularly in the intestine. Indeed, in a mouse model of intestinal tumorigenesis, the number of tumor was reduced by the ablation of c-Jun or the mutation of JNK phosphorylation sites on c-Jun, suggesting that JNK participates in the transformation process in the intestine [28]. Consistent with this, JNK also promotes colorectal carcinogenesis under high-fat dietary conditions [14]. Furthermore, high levels of JNK expression are found in colon cancer [29]. Our study further underlines the role of JNK in colorectal cancer cell growth, as the inhibition of JNK in LS174T colon cancer cells reduced cell proliferation *in vitro* and tumor xenograft growth *in vivo*. Finally, since mTOR signaling pathway is also involved in colon cancer progression [30–32], our observation that the combined JNK and mTOR inhibition had synergistic effects might be particularly relevant in the context of colon cancer.

In conclusion, our study shows that the anticancer efficacy of rapamycin can be potentiated by the simultaneous inhibition of JNK signaling pathway, and may provide a novel strategy in cancer therapy.

ACKNOWLEDGMENT

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