Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 331 (2005) 295–302
BBRC
www.elsevier.com/locate/ybbrc

The rapamycin analog CCI-779 is a potent inhibitor of pancreatic cancer cell proliferation ☆

Takayuki Asano [1], Yixin Yao [1], Jijiang Zhu, Donghui Li, James L. Abbruzzese, Shrikanth A. Reddy *

Department of Gastrointestinal Medical Oncology, Unit 426, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

Received 18 March 2005
Available online 2 April 2005

Abstract

We present immunohistochemical evidence that the mTOR/p70s6k pathway is activated in pancreatic tumors and show that the mTOR inhibitor and rapamycin analog CCI-779 potently suppresses the proliferation of pancreatic cancer cells. Consistent with a recent study, CCI-779 increased c-Jun phosphorylation (Ser63) in a dose- and time-dependent manner, and induced apoptosis in p53-defective BxPC-3 cells. In contrast to the study, however, we observed that CCI-779 concomitantly increased c-Jun protein levels and that its ability to induce apoptosis might not require the activated c-Jun. Furthermore, CCI-779 neither induced c-Jun phosphorylation in other p53-defective pancreatic cancer cells (MiaPaCa-2) nor inhibited their proliferation. c-Jun, in fact, appeared to be partly responsible for the resistance of MiaPaCa-2 cells to CCI-779. Together, these results indicate a complex role for c-Jun in cellular responses to CCI-779 and provide an important basis for investigating CCI-779 further as a potential therapeutic agent for pancreatic tumors.

Keywords: CCI-779; Rapamycin; Pancreatic cancer; p70s6k; Akt; PI 3-kinase; c-Jun; Drug resistance

Pancreatic cancer is one of the leading causes of cancer-related mortality in the western world. Conventional strategies such as chemotherapy and radiotherapy have not improved the median survival time of patients with metastatic disease for the last 30 years [1]. Given this dismal record, attention has turned to molecular therapeutics as a powerful new approach for targeting proteins implicated in pancreatic cancer initiation and progression. The goal is to accelerate the discovery of novel drugs to use in pancreatic cancer therapy [2,3].

Rapamycin is a macrolide fungicide that has demonstrated impressive anti-tumor activity [4]. It also possesses potent anti-microbial and immunosuppressant properties, and inhibits the translation of proteins required for cell-cycle progression from G_1 to S phase. A rapamycin analog (ester) known as CCI-779 has been developed in an effort to obtain more favorable pharmaceutical, toxicologic, and anti-tumor profiles in preclinical evaluations than those achieved by rapamycin [4,5]. Inhibition of rapamycin-sensitive signaling pathways by CCI-779 appears to be the basis for its potent activity against a wide range of human tumors in tissue culture and xenograft models [4,5]. Like rapamycin, it binds intracellularly to the immunophilin FK506 binding protein (FKBP12) and forms a complex that inhibits the

☆ *Abbreviations:* mTOR, mammalian target of rapamycin; p70s6k, p70 ribosomal S6 protein kinase, PI 3-kinase, phosphoinositide 3-kinase.

* Corresponding author. Fax: +1 713 745 1163.
E-mail address: sa08366@odin.mdacc.tmc.edu (S.A. Reddy).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.166

activity of mammalian target of rapamycin (mTOR) or FRAP, a protein kinase that belongs to the phosphoinositide 3-kinase (PI 3-kinase) super-family [4,6]. When mTOR is inhibited, its signals such as those that induce the phosphorylation of the 70 kDa, 40S ribosomal protein kinase (p70s6k), and the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) are blocked, leading to G1 arrest in most cell types [7,8] and to p53-independent apoptosis in some others [9–11]. The mechanism by which rapamycin induces apoptosis in cells lacking functional p53 under serum-free conditions was recently shown to involve 4E-BP1 and the ASK/JNK/Jun pathway [11].

There is considerable evidence that mTOR functions in the PI 3-kinase pathway downstream of Akt [12–16]. Consistent with a role for mTOR in tumorigenesis, both PI 3-kinase and Akt as well as the PTEN gene, a lipid phosphatase [17] which functions as their natural antagonist in normal cells, have been strongly implicated in human cancer [18–20]. It has been shown that tumors that depend on activated PI 3-kinase/Akt such as those with defective PTEN function are highly sensitive to mTOR inhibitors [21–23]. Various studies, including our own, have demonstrated that the PI 3-kinase pathway is activated in pancreatic adenocarcinoma and that it is important for the survival, proliferation, and drug resistance of pancreatic cancer cells [24–31]. We also showed that PTEN expression was significantly reduced in over 60% of the pancreatic tumor tissues and cell lines we examined [31]. In addition to PTEN, a large proportion of pancreatic tumor tissues and cell lines also lack functional p53 [32,33]. We therefore investigated the activation status of mTOR in pancreatic normal and tumor tissue specimens, and the effects of CCI-779 on specific cell lines. Our results indicate that mTOR is activated in pancreatic tumors and that CCI-779 is a potent inhibitor of growth for many if not all pancreatic cancer cell lines that contained defective p53. However, while CCI-779 induced c-Jun phosphorylation in a sensitive cell line consistent with a previous study [11], its ability to induce apoptosis did not seem to require the activated c-Jun. Furthermore, c-Jun appeared to partly account for the resistance of at least one pancreatic cancer cell line to CCI-779. Together, these data suggest that CCI-779 deserves further investigation as a potential therapeutic agent for pancreatic cancer treatment.

Materials and methods

Materials. LY294002 (PI 3-kinase inhibitor) and SP600125 (c-Jun N-terminal kinase or JNK inhibitor) were obtained from Biomol Research Laboratories, and rapamycin (mTOR inhibitor) was purchased from Calbiochem. Akt1/2, JNK, p65RelA, c-Jun, and anti-phospho-c-Jun (serine 63) antibodies were obtained from Santa Cruz Biotechnology whereas phospho-Akt (Ser473), p70s6k, and phospho-p70s6k (Thr389) antibodies were from Cell Signaling Technology. Anti-PARP antibodies were purchased from BD Pharmingen. Tissue sections were obtained from the NCI Cooperative Human Tissue Network.

Cell culture. AsPC-1, Panc-1, Capan-1, MIA PaCa-2, and BxPC-3 cells were purchased from the American Type Culture Collection and cultured as described in their product information sheets. Panc-3, Panc-28, and Panc-48 cells were provided by Drs. Paul Chiao and Keping Xie (M.D. Anderson Cancer Center), and maintained in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum under standard culture conditions.

Immunoblotting analysis. Serum-starved (16 h) pancreatic cancer cells were lysed and whole-cell extracts (WCE) were prepared as described previously [34]. Where indicated, cells were treated with vehicle (control), the PI 3-kinase inhibitor LY294002 or the mTOR inhibitors rapamycin or CCI-779, and washed with ice-cold phosphate-buffered saline before WCE preparation. WCE were clarified by centrifugation and proteins were resolved by SDS–PAGE. Following protein transfer, nitrocellulose membranes were probed for total and phosphorylated p70s6k, total and phosphorylated Akt, and actin. Specific bands were detected by ECL (Amersham-Pharmacia Biotech). For the detection of poly(ADP)-ribose polymerase (PARP) cleavage, total c-Jun, phospho-c-Jun, JNK, or p65RelA, the pellet that remained after WCE preparation was further extracted and protein was combined with WCE as described previously [34]. This protein mixture was resolved by SDS–PAGE and probed by Western blotting as described above.

Immunohistochemistry. The phosphorylation status of p70s6k was investigated in paraffin sections of residual surgical tissues from four different patients with ductal pancreatic adenocarcinoma. These sections were heated at 65 °C overnight, deparaffinized in xylene, and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. Sections were then blocked with 3% bovine serum albumin and normal horse serum at 37 °C for 30 min, and then overnight with phospho-p70s6k (Thr389) antibody at 4 °C in a humidified chamber, and finally with a biotinylated secondary antibody for 30 min at 37 °C. The antibody complex was detected by avidin–biotin–peroxidase complex solution and visualized by 3,3′-diaminobenzidine (Zymed Laboratories). Sections were counterstained with hematoxylin for 5 min and mounted with Eukit (Calibrated Instrument). Immunostaining was observed under a light microscope, and a semiquantitative score for intensity was given to the different samples: strong, moderate, or weak, relative to the staining observed for smooth muscle cells in the vascular wall and the intestinal wall.

Proliferation assays. Pancreatic cancer cells were plated at a density of 5×10^3 cells per well in 96-well culture plates. After 16 h, the medium was removed, and the cells were cultured in fresh serum-containing medium in the presence or absence of CCI-779, TGFβ, and SP600125. At various times, cells were stained with crystal violet to determine the absorbance at 540 nm in a Packard plate reader. Each data point was obtained in triplicate, and averages and standard deviation were estimated.

Soft-agar assays. Cells (1×10^4) were mixed with 1 ml of a 0.33% Noble agar solution and added on top of 1 ml of a solidified layer of 0.5% agarose in 12-well culture plates. Fresh inhibitors were added every 3 days, and colony formation was monitored biweekly for 3 weeks. Assays were performed in triplicate and colonies were photographed using a Nikon Eclipse TE2000S Inverted microscope and a Hamamatsu digital camera.

Stable expression of TAM67 in MiaPaCa-2 cells. MiaPaCa-2 cells were cultured in DMEM containing 10% FCS, 1000 U/ml penicillin–streptomycin, and 2 mM glutamine, and transfected with the empty pcDNA3.1 vector or with the pcDNA3.1-TAM67 plasmid using the FuGene reagent (Roche). After 24 h, transfected cells were incubated with 400 μg/ml G418. Drug-resistant colonies were expanded and employed for proliferation assays to test the effects of TGFβ and CCI-779.

Results

mTOR is activated in pancreatic tumor tissues and cell lines

We investigated the phosphorylation status of p70s6k as a measure of mTOR activation in eight different serum-starved pancreatic cancer cell lines. Immunoblotting analysis showed that p70s6k was phosphorylated on Thr389, an mTOR-modified residue that is critical for its kinase activity, and that the levels of phosphorylated enzyme varied substantially between cell lines (Fig. 1A). These data indicate that pancreatic cancer cells contain activated mTOR and p70s6k, consistent with previous studies on Panc-1, BxPC-3, and MiaPaCa-2 cells [29,30]. For confirmation, we next examined normal and tumor tissue specimens from pancreatic cancer patients. Immunohistochemical analysis indicated that pancreatic tumor tissues indeed contained higher levels of phosphorylated p70s6k (p-p70s6k) than normal controls (Fig. 1B) and that three of the four pancreatic, adenocarcinoma samples examined were strongly positive for p-p70s6k.

mTOR inhibitor CCI-779 blocks anchorage-dependent and -independent growth of pancreatic cancer cells

A number of important studies have shown that p70s6k is regulated in response to multiple signaling inputs [35]. There is also compelling evidence that it is an important target of the PI 3-kinase pathway and that PI 3-kinase/Akt-induced p70s6k activation is mediated by mTOR [13–16]. Thus, the mechanism of p70s6k activation in this signaling cascade is sensitive to both mTOR (rapamycin) and PI 3-kinase (wortmannin, LY294002) inhibitors and involves p70s6k phosphorylation on Thr389 [35]. Confirming the essential role of PI 3-kinase and mTOR in pancreatic cancer cells, the levels of p-p70s6k were potently inhibited by LY294002, rapamycin, and the rapamycin analog CCI-779 (Fig. 2A). Akt-Ser473 phosphorylation was inhibited by LY294002 but not by CCI-779 or rapamycin consistent with the notion that mTOR is downstream of Akt in the PI 3-kinase signaling pathway (Fig. 2A). The inhibition of p70s6k phosphorylation was not due to a reduction in p70s6k protein levels which were unaffected by inhibitor treatment.

We next investigated if the pharmacological inactivation of mTOR by CCI-779 would affect the proliferation of pancreatic cancer cells under two different conditions. The ability of different pancreatic cancer cell lines to proliferate under serum-induced, anchorage-dependent conditions was investigated over a period of 4 days. CCI-779 potently inhibited the proliferation of four different cell lines, with Capan-1 and BxPC-3 being slightly more sensitive (over 90% inhibition, Fig. 2B) than Panc-1 and AsPC-1.

Since transformed cells and cells derived from tumors grow in anchorage-independent conditions, we investigated if CCI-779 also affected the ability of pancreatic cancer cells to form colonies in agarose medium. Compared to vehicle-treated controls, the ability of cells to grow in soft agar was potently inhibited when cultured in medium containing CCI-779 (Fig. 2C). Consistent with previous studies, colony formation was strongly inhibited by rapamycin and LY294002 as well.

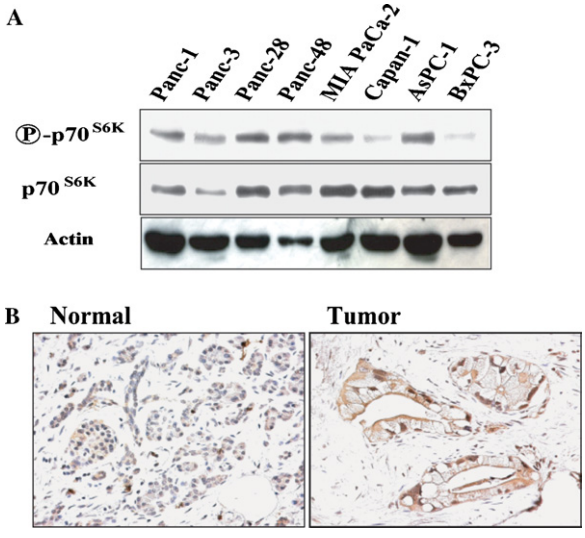

Fig. 1. p70s6k is phosphorylated in pancreatic tumor cell lines and tissues. (A) Whole-cell extracts were prepared from various serum-starved (16 h) pancreatic cancer cell lines and analyzed by immunoblotting with antibodies that specifically recognized total p70s6k or its phosphorylated form (Thr389). (B) Immunohistochemical staining. A representative section from the four different pancreatic adenocarcinoma tissue specimens (tumor) examined is shown. Residual normal adjacent tissues were present in the sample and pancreatic epithelial cells showed weak to moderate staining (normal).

CCI-779 induces c-Jun expression and apoptosis in BxPC-3 cells

Huang et al. [11] recently demonstrated that rapamycin increases c-Jun phosphorylation, without altering its protein levels, in p53-defective, serum-deprived cells and thus induces apoptosis. We, therefore, investigated the effect of CCI-779 on pancreatic cancer cell lines many of which lack functional p53. Indeed, CCI-779 at concentrations as low as 2 pM was able to potently induce the phosphorylation/activation of c-Jun in BxPC-3 cells (Fig. 3A). However, in contrast to the observations of Huang et al. [11], CCI-779 was concomitantly also able to increase c-Jun protein levels over a wide range of concentrations (Fig. 3A). These effects of CCI-779 were observed both in the absence and presence of serum (Fig. 3A) within 2 h of treatment (Fig. 3B), and appeared to be specific because no changes were observed for other proteins such as p65RelA and JNK.

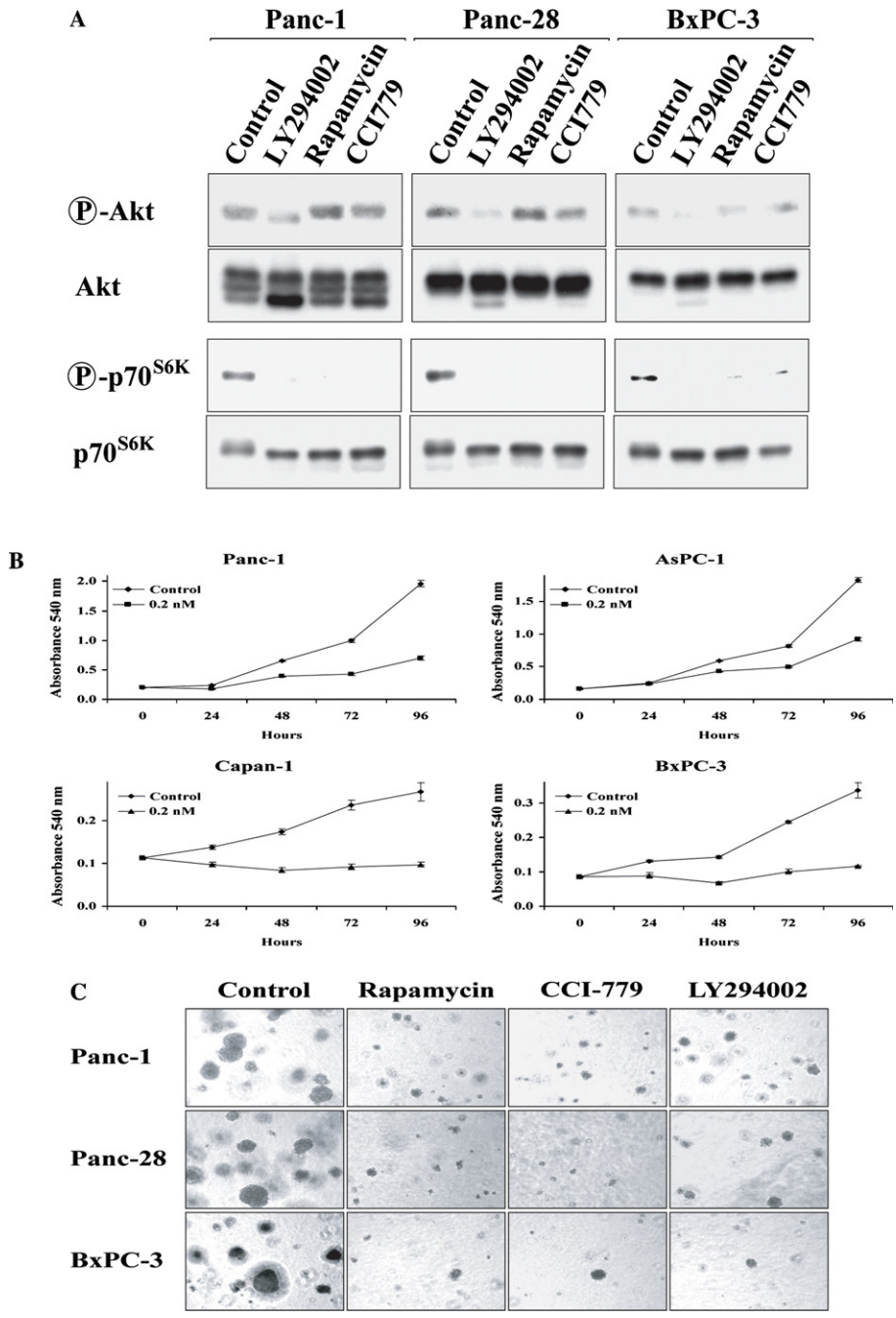

Fig. 2. CCI-779 is a potent inhibitor of mTOR and pancreatic cancer cell growth. (A) Serum-starved cells were either treated with LY294002 (20 μM), rapamycin (0.25 μM), or CCI-779 (0.2 nM) for 2 h or left untreated (control). Whole-cell extracts were then prepared and analyzed by immunoblotting analysis for Akt, p70s6k, and their phosphorylated forms. (B) The effect of CCI-779 on the proliferation (anchorage-dependent) of pancreatic cancer cells was determined by crystal-violet staining (540 nm). (C) Cells were treated with CCI-779, rapamycin, or LY294002 to test their ability to grow in soft agar (anchorage-independent conditions).

Since rapamycin induces apoptosis in p53-defective cells, we investigated the effect of CCI-779 on serum-starved BxPC-3 cells using PARP ((poly)ADP-ribose polymerase) as a marker. PARP is typically cleaved from a 116 kDa protein to a smaller 85 kDa form in cells undergoing apoptosis. Indeed, PARP was completely cleaved in BxPC-3 cells within 8–24 h of treatment (Fig. 3C), indicating that CCI-779, like rapamycin, triggers apoptosis in p53-deficient serum-deprived cells. Surprisingly, however, pre-treatment of BxPC-3 cells with an inhibitor (SP600125) that blocked c-Jun phosphorylation/activation (data not shown) neither induced PARP cleavage on its own nor appeared to interfere with the ability of CCI-779 to trigger apoptosis (Fig. 3C).

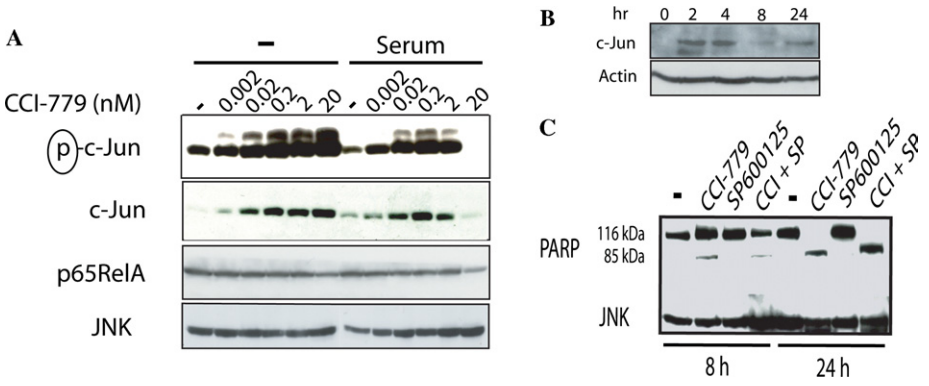

Fig. 3. CCI-779 induces apoptosis independently of c-Jun in BxPC-3 cells. (A) BxPC-3 cells cultured in the absence (16 h) or presence of serum were treated, as indicated, with various concentrations of CCI-779. Whole-cell extracts were prepared, subjected to SDS–PAGE, and analyzed by immunoblotting with antibodies that recognized phospho-c-Jun (Ser63), c-Jun, p65RelA or JNK. (B) Whole-cell extracts from BxPC-3 cells treated with 0.2 nM CCI-779 for the indicated time points were analyzed by Western blotting for c-Jun and actin. (C) BxPC-3 cells were treated with or without 2 nM CCI-779. Where indicated, the JNK inhibitor SP600125 (10 μM) was added to cells 30 min prior to the addition of CCI-779. After the addition of CCI-779, cells were harvested after 8 or 24 h and whole-cell extracts were prepared for immunoblotting analysis of PARP and JNK expression. PARP is a 116 kDa polypeptide that is typically degraded in apoptotic cells to an 85 kDa form.

Resistance of MiaPaCa-2 cells to CCI-779 treatment is reduced by a c-Jun transactivation mutant (TAM67)

CCI-779 did not induce the phosphorylation or expression of c-Jun in MiaPaCa-2 pancreatic cancer cells (Fig. 4A). Significantly, MiaPaCa-2 was also resistant to CCI-779 treatment unlike the BxPC-3 cells (Fig. 4B). However, because MiaPaCa-2 expressed much higher levels of c-Jun than BxPC-3 cells (data not shown), we investigated the possibility that c-Jun was, in fact, the cause of their resistance to CCI-779. MiaPaca-2 cells stably expressing a c-Jun transactivation mutant (TAM67) were therefore generated to test the possibility and, to our surprise, found to be more sensitive to CCI-779 treatment than those that had been transfected with empty vector (Fig. 4C). In sharp contrast, TAM67 expression did not sensitize MiaPaCa-2 cells to TGF-β treatment.

Discussion

Using cell lines and tumor tissue specimens, we show that the Akt/mTOR/p70s6k pathway is constitutively activated in pancreatic cancer. Our data also indicated that CCI-779 blocked this pathway and that it potently inhibited the serum-induced proliferation of various pancreatic cancer cell lines in anchorage-dependent and -independent conditions. Trypan blue exclusion assays and immunoblotting analysis of PARP cleavage showed that greater than 95% of pancreatic cancer cells were viable under these conditions suggesting that CCI-779 did not induce apoptosis in the presence of serum (data not shown). Previous studies utilized fluorescence-activated cell sorting analysis to show that rapamycin induced a G_0–G_1 cell-cycle arrest and inhibited the serum-induced proliferation of a fraction of Panc-1 and BxPC-3 cells [30]. Our data suggest, in comparison, that CCI-779 is likely to be a far more effective inhibitor of proliferation and that it induces apoptosis in serum-deprived, p53-defective BxPC-3 cells. Huang et al. [11] recently showed that rapamycin-induced apoptosis that is observed in p53-deficient cells was dependent on the ability of rapamycin to induce c-Jun phosphorylation/activation. Our data indicate that CCI-779 was capable of inducing c-Jun phosphorylation in BxPC-3 cells but that it also

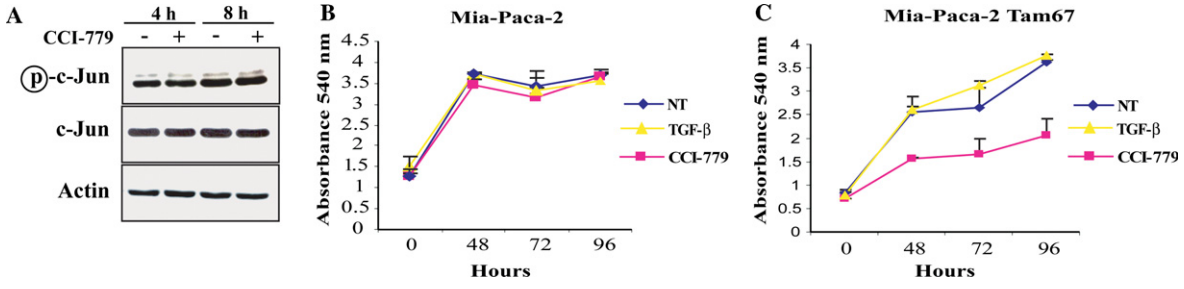

Fig. 4. MiaPaCa-2 cells are resistant to CCI-779 due to a c-Jun-regulated mechanism. (A) Whole-cell extracts of MiaPaCa-2 cells treated in the absence or presence of 2 nM CCI-779 for the indicated time points were analyzed by Western blotting for phospho-c-Jun (Ser63), c-Jun or actin. MiaPaCa-2 cells stably transfected with empty vector (B) or the c-Jun TAM67 mutant (C) were treated without (NT) or with CCI-779 (0.2 nM) or TGF-β. At the indicated time points, crystal violet assays were performed and the absorbance was determined at 540 nm in a Packard plate reader.

increased c-Jun protein levels. Furthermore, we showed that phospho-c-Jun might not mediate CCI-779-induced apoptosis raising questions about its role in CCI-779 action on BxPC-3 cells. Not all pancreatic cancer cells might be sensitive, since the serum-induced proliferation of MiaPaCa-2 cells was unaffected by a similar dose of CCI-779. To our surprise, the resistance of MiaPaCA-2 cells to CCI-779 was significantly lowered by a Jun mutant. The role of Jun in the resistance of cancer cells to drugs is well documented [36].

CCI-779 is currently being investigated in various clinical studies in patients with solid tumors and appears to be well tolerated at doses that exhibit potent anti-tumor activity against different types of refractory neoplasms [4,5,37,38]. Recent studies have indicated that tumors with certain characteristics would be more responsive to CCI-779 than others [21–23]. Indeed, CCI-779 reduced neoplastic proliferation and tumor size in PTEN $^{+/-}$ mice and preferentially blocked growth of PTEN-deficient cancer cells in vitro and in vivo. In contrast to its effects on PTEN-deficient cells, CCI-779 treatment did not affect the growth of mouse embryonic fibroblasts that contained wild-type PTEN. Although PTEN may not be mutated or deleted [39,40], we [31], and others [41], have observed that its expression is frequently either reduced or lost in pancreatic cancer. It is unclear nonetheless, if PTEN is a factor in the sensitivity of pancreatic cancer cells to CCI-779, because even though Panc-1, Capan-1, and AsPC-1 expressed PTEN at a substantially higher level than the BxPC-3 [31], they were all equally sensitive to 0.2 nM CCI-779. A more detailed study is underway to test if these cell lines are differentially sensitive to CCI-779 in a PTEN-dependent manner.

There are indications that a wider variety of tumors than those lacking functional PTEN respond to CCI-779 treatment and that cancer cells overexpressing PI 3-kinase or Akt, for example, are also sensitive [2]. In addition to differences in PTEN expression, the four cell lines we examined differed in the extent to which p70s6k was phosphorylated (Fig. 1A) and Cheng et al. [42], have reported that the AKT2 gene is amplified and overexpressed in the Panc-1 and AsPC-1 cell lines, and that it contributes to the malignant phenotype in 10% of pancreatic carcinomas. Compelling evidence has been presented elsewhere that Akt-mediated sensitivity of glioblastoma and prostate cancer cells to rapamycin and CCI-779 is due to the ability of both inhibitors to downregulate cyclin D1 and c-Myc [43]. Surprisingly, rapamycin did not affect cyclin D1 and c-Myc levels in AsPC-1 and BxPC-3 cells in our previous study [31], and yet both cell lines were highly sensitive to CCI-779 treatment (Fig. 2B). It is pertinent to note that activated Ki-Ras does not appear to be influencing the sensitivity of pancreatic cancer cells to CCI-779 because cells expressing wild-type Ki-Ras (BxPC-3) were inhibited almost as potently as the others that harbored the oncogenic form. In conclusion, the responsiveness of pancreatic cancer cells to CCI-779 could be due to the interplay of a complex set of factors that remain to be identified.

Together, these data indicate that CCI-779 potently blocks the growth of pancreatic cancer cells and establish the basis for evaluating it further as a potential therapeutic agent for treating pancreatic cancer patients.

Acknowledgments

We are grateful to Dr. Phil Frost and Wyeth Ayerst for generously providing us CCI-779, to Dr. Michael Birrer for TAM67, and to Walter Pagel for critically reading the manuscript. This work was supported by grants to SAR from the Lustgarten Foundation for Pancreatic Cancer Research, by funds from the University Cancer Foundation at the University of Texas M.D. Anderson Cancer Center, the Topfer Fund for Pancreatic Cancer Research, and the NCI SPORE CA101936 in Pancreatic Cancer. We received valuable assistance from the DNA sequencing and Media preparation facilities at the University of Texas M.D. Anderson Cancer Center that are supported by a grant from the National Cancer Institute (CA016672).

References

[1] D.B. Evans, J.E. Lee, P.W. Pisters, C. Charnsangavej, L.M. Ellis, P.J. Chiao, R. Lenzi, J.L. Abbruzzese, Advances in the diagnosis and treatment of adenocarcinoma of the pancreas, Cancer Treat Res. 90 (1997) 109–125.
[2] G.B. Mills, Y. Lu, E.C. Kohn, Linking molecular therapeutics to molecular diagnostics: inhibition of the FRAP/RAFT/TOR component of the PI3K pathway preferentially blocks PTEN mutant cells in vitro and in vivo, Proc. Natl. Acad. Sci. USA 98 (2001) 10031–10033.
[3] R.A. Wolff, Exploiting molecular targets in pancreatic cancer, Hematol. Oncol. Clin. North Am. 16 (2002) 139–157.
[4] M. Hidalgo, E.K. Rowinsky, The rapamycin-sensitive signal transduction pathway as a target for cancer therapy, Oncogene 19 (2000) 6680–6686.
[5] M.W. Harding, Immunophilins, mTOR, and pharmacodynamic strategies for a targeted cancer therapy, Clin. Cancer Res. 9 (2003) 2882–2886.
[6] R.T. Abraham, Identification of TOR signaling complexes: more TORC for the cell growth engine, Cell 111 (2002) 9–12.
[7] S. Muthukkumar, T.M. Ramesh, S. Bondada, Rapamycin, a potent immunosuppressive drug, causes programmed cell death in B lymphoma cells, Transplantation 60 (1995) 264–270.
[8] T. Seufferlein, E. Rozengurt, Rapamycin inhibits constitutive p70s6k phosphorylation, cell proliferation, and colony formation in small cell lung cancer cells, Cancer Res. 56 (1996) 3895–3897.
[9] H. Hosoi, M.B. Dilling, T. Shikata, L.N. Liu, L. Shu, R.A. Ashmun, G.S. Germain, R.T. Abraham, P.J. Houghton,

Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells, Cancer Res. 59 (1999) 886–894.
[10] Y. Shi, A. Frankel, L.G. Radvanyi, L.Z. Penn, R.G. Miller, G.B. Mills, Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro, Cancer Res. 55 (1995) 1982–1988.
[11] S. Huang, L. Shu, M.B. Dilling, J. Easton, F.C. Harwood, H. Ichijo, P.J. Houghton, Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21(Cip1), Mol. Cell 11 (2003) 1491–1501.
[12] J. Downward, Mechanisms and consequences of activation of protein kinase B/Akt, Curr. Opin. Cell Biol. 10 (1998) 262–267.
[13] P.H. Scott, G.J. Brunn, A.D. Kohn, R.A. Roth, J.C. Lawrence Jr., Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway, Proc. Natl. Acad. Sci USA 95 (1998) 7772–7777.
[14] B.T. Nave, M. Ouwens, D.J. Withers, D.R. Alessi, P.R. Shepherd, Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation, Biochem. J. 344 (Pt. 2) (1999) 427–431.
[15] L. Hu, C. Zaloudek, G.B. Mills, J. Gray, R.B. Jaffe, In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002), Clin. Cancer Res. 6 (2000) 880–886.
[16] A. Sekulic, C.C. Hudson, J.L. Homme, P. Yin, D.M. Otterness, L.M. Karnitz, R.T. Abraham, A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells, Cancer Res. 60 (2000) 3504–3513.
[17] T. Maehama, J.E. Dixon, The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate, J. Biol. Chem. 273 (1998) 13375–13378.
[18] I. Vivanco, C.L. Sawyers, The phosphatidylinositol 3-kinase AKT pathway in human cancer, Nat. Rev. Cancer 2 (2002) 489–501.
[19] P.A. Steck, M.A. Pershouse, S.A. Jasser, W.K. Yung, H. Lin, A.H. Ligon, L.A. Langford, M.L. Baumgard, T. Hattier, T. Davis, C. Frye, R. Hu, B. Swedlund, D.H. Teng, S.V. Tavtigian, Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, Nat. Genet. 15 (1997) 356–362.
[20] J. Li, C. Yen, D. Liaw, K. Podsypanina, S. Bose, S.I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S.H. Bigner, B.C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M.H. Wigler, R. Parsons, PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer, Science 275 (1997) 1943–1947.
[21] M.S. Neshat, I.K. Mellinghoff, C. Tran, B. Stiles, G. Thomas, R. Petersen, P. Frost, J.J. Gibbons, H. Wu, C.L. Sawyers, Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR, Proc. Natl. Acad. Sci. USA 98 (2001) 10314–10319.
[22] K. Podsypanina, R.T. Lee, C. Politis, I. Hennessy, A. Crane, J. Puc, M. Neshat, H. Wang, L. Yang, J. Gibbons, P. Frost, V. Dreisbach, J. Blenis, Z. Gaciong, P. Fisher, C. Sawyers, L. Hedrick-Ellenson, R. Parsons, An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/− mice, Proc. Natl. Acad. Sci. USA 98 (2001) 10320–10325.
[23] K. Yu, L. Toral-Barza, C. Discafani, W.G. Zhang, J. Skotnicki, P. Frost, J.J. Gibbons, mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer, Endocr. Relat. Cancer 8 (2001) 249–258.
[24] S.S.W. Ng, M.S. Tsao, S. Chow, D.W. Hedley, Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells, Cancer Res. 60 (2000) 5451–5455.
[25] S.S. Ng, M.S. Tsao, T. Nicklee, D.W. Hedley, Wortmannin inhibits pkb/akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice, Clin. Cancer Res. 7 (2001) 3269–3275.
[26] R.A. Perugini, T.P. McDade, F.J. Vittimberga Jr., M.P. Callery, Pancreatic cancer cell proliferation is phosphatidylinositol 3-kinase dependent, J. Surg Res. 90 (2000) 39–44.
[27] V.M. Bondar, B. Sweeney-Gotsch, M. Andreeff, G.B. Mills, D.J. McConkey, Inhibition of the phosphatidylinositol 3'-kinase-AKT pathway induces apoptosis in pancreatic carcinoma cells in vitro and in vivo, Mol. Cancer Ther. 1 (2002) 989–997.
[28] Z. Yao, Y. Okabayashi, Y. Yutsudo, T. Kitamura, W. Ogawa, M. Kasuga, Role of Akt in growth and survival of PANC-1 pancreatic cancer cells, Pancreas 24 (2002) 42–46.
[29] M. Grewe, F. Gansauge, R.M. Schmid, G. Adler, T. Seufferlein, Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6K pathway in human pancreatic cancer cells, Cancer Res. 59 (1999) 3581–3587.
[30] S.A. Shah, M.W. Potter, R. Ricciardi, R.A. Perugini, M.P. Callery, FRAP-p70s6K signaling is required for pancreatic cancer cell proliferation, J. Surg. Res. 97 (2001) 123–130.
[31] T. Asano, Y. Yao, J. Zhu, D. Li, J.L. Abbruzzese, S.A. Reddy, The PI 3-kinase/Akt signaling pathway is activated due to aberrant Pten expression and targets transcription factors NF-kappaB and c-Myc in pancreatic cancer cells, Oncogene 23 (2004) 8571–8580.
[32] G. Casey, Y. Yamanaka, H. Friess, M.S. Kobrin, M.E. Lopez, M. Buchler, H.G. Beger, M. Korc, p53 mutations are common in pancreatic cancer and are absent in chronic pancreatitis, Cancer Lett. 69 (1993) 151–160.
[33] N.S. Pellegata, F. Sessa, B. Renault, M. Bonato, B.E. Leone, E. Solcia, G.N. Ranzani, K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions, Cancer Res. 54 (1994) 1556–1560.
[34] D. Koul, Y. Yao, J.L. Abbruzzese, W.K. Yung, S.A. Reddy, Tumor suppressor MMAC/PTEN inhibits cytokine-induced NFkappaB activation without interfering with the IkappaB degradation pathway, J. Biol. Chem. 276 (2001) 11402–11408.
[35] A. Dufner, G. Thomas, Ribosomal S6 kinase signaling and the control of translation, Exp. Cell Res. 253 (1999) 100–109.
[36] I. Vasilevskaya, P.J. O'Dwyer, Role of Jun and Jun kinase in resistance of cancer cells to therapy, Drug Resist. Update 6 (2003) 147–156.
[37] J.M. Peralba, L. DeGraffenried, W. Friedrichs, L. Fulcher, V. Grunwald, G. Weiss, M. Hidalgo, Pharmacodynamic evaluation of CCI-779, an inhibitor of mTOR, in cancer patients, Clin. Cancer Res. 9 (2003) 2887–2892.
[38] J.P. Dutcher, Mammalian target of rapamycin (mTOR) inhibitors, Curr. Oncol. Rep. 6 (2004) 111–115.
[39] A. Sakurada, A. Suzuki, M. Sato, H. Yamakawa, K. Orikasa, S. Uyeno, T. Ono, N. Ohuchi, S. Fujimura, A. Horii, Infrequent genetic alterations of the PTEN/MMAC1 gene in Japanese patients with primary cancers of the breast, lung, pancreas, kidney, and ovary, Jpn. J. Cancer Res. 88 (1997) 1025–1028.
[40] K. Okami, L. Wu, G. Riggins, P. Cairns, M. Goggins, E. Evron, N. Halachmi, S.A. Ahrendt, A.L. Reed, W. Hilgers, S.E. Kern, W.M. Koch, D. Sidransky, J. Jen, Analysis of PTEN/MMAC1 alterations in aerodigestive tract tumors, Cancer Res. 58 (1998) 509–511.
[41] M.P. Ebert, G. Fei, L. Schandl, C. Mawrin, K. Dietzmann, P. Herrera, H. Friess, T.M. Gress, P. Malfertheiner, Reduced PTEN expression in the pancreas overexpressing transforming growth factor-beta 1, Br. J. Cancer 86 (2002) 257–262.

[42] J.Q. Cheng, B. Ruggeri, W.M. Klein, G. Sonoda, D.A. Altomare, D.K. Watson, J.R. Testa, Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA, Proc. Natl. Acad. Sci. USA 93 (1996) 3636–3641.

[43] J.F. Gera, I.K. Mellinghoff, Y. Shi, M.B. Rettig, C. Tran, J.H. Hsu, C.L. Sawyers, A.K. Lichtenstein, AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression, J. Biol. Chem. 279 (2004) 2737–2746.