Combined Inhibition of Cyclin-Dependent Kinases (Dinaciclib) and AKT (MK-2206) Blocks Pancreatic Tumor Growth and Metastases in Patient-Derived Xenograft Models

Chaoxin Hu1,2, Tikva Dadon3, Venugopal Chenna1,2, Shinichi Yabuuchi1, Rajat Bannerji4, Robert Booher4, Peter Strack4, Nilofer Azad3, Barry D. Nelkin3, and Anirban Maitra1,2,3,5

Abstract

KRAS is activated by mutation in the vast majority of cases of pancreatic cancer; unfortunately, therapeutic attempts to inhibit KRAS directly have been unsuccessful. Our previous studies showed that inhibition of cyclin-dependent kinase 5 (CDK5) reduces pancreatic cancer growth and progression, through blockade of the centrally important RAL effector pathway, downstream of KRAS. In the current study, the therapeutic effects of combining the CDK inhibitor dinaciclib (SCH727965; MK-7965) with the pan-AKT inhibitor MK-2206 were evaluated using orthotopic and subcutaneous patient-derived human pancreatic cancer xenograft models. The combination of dinaciclib (20 mg/kg, i.p., three times a week) and MK-2206 (60 mg/kg, orally, three times a week) dramatically blocked tumor growth and metastasis in all eight pancreatic cancer models examined. Remarkably, several complete responses were induced by the combination treatment of dinaciclib and MK-2206. The striking results obtained in these models demonstrate that the combination of dinaciclib with the pan-AKT inhibitor MK-2206 is promising for therapeutic evaluation in pancreatic cancer, and strongly suggest that blocking RAL in combination with other effector pathways downstream from KRAS may provide increased efficacy in pancreatic cancer. Based on these data, an NCI–CTEP-approved multicenter phase I clinical trial for pancreatic cancer of the combination of dinaciclib and MK-2206 (NCT01783171) has now been opened. Mol Cancer Ther; 14(7); 1532–9. ©2015 AACR.

Corresponding Authors: Barry D. Nelkin, Johns Hopkins University School of Medicine, CRB1 Room 552, 1650 Orleans Street, Baltimore, MD 21287. Phone: 410-745-0861; Fax: 410-745-3375; E-mail: bnelkin@jhmi.edu; and Anirban Maitra, Department of Pathology, The University of Texas MD Anderson Cancer Center, Unit 0085, Room 3916, Houston, TX 77030. Phone: 713-745-3375; E-mail: ama@mdanderson.org


Small Molecule Therapeutics

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States (1). Despite concerted research efforts, there has been little improvement in PDAC prognosis over the last several decades, and the 5-year survival rate of pancreatic cancer remains below 6% (2). In addition, pancreatic cancer incidence continues to increase; from 2006 to 2010, rates increased by 1.3% per year (1). Therefore, there is an urgent need to find effective systemic therapies to treat this highly lethal cancer.

Activating mutations in KRAS are found in more than 90% of patients with pancreatic cancer (3, 4). A series of in vivo evidence shows that mutant KRAS is a driver for tumor initiation and progression in PDAC (5–9). Thus, oncogenic KRAS is considered a prime therapeutic target for pancreatic cancer. Unfortunately, therapeutic attempts to inhibit mutant KRAS thus far have been unsuccessful (10). A promising alternative strategy has been to target KRAS downstream effector pathways. KRAS has several effector pathways, notably including the PI3K/AKT, RAF/MEK/ERK, and RAL effector pathways. Activation of the PI3K/AKT and RAF/MEK/ERK pathways is very common in pancreatic cancer, and these pathways appear to be important to pancreatic cancer growth (6, 10, 11). Combined inhibition of these pathways has been shown to synergistically inhibit pancreatic cancer growth in preclinical models (11–13), and clinical trials to simultaneously inhibit these two pathways are in progress.

Importantly, Counter and colleagues (14, 15) have shown that, among the KRAS effector pathways, the RAL pathway is especially critical for the development of pancreatic cancer. This strongly suggests that inhibiting the RAL pathway is a promising central target for blocking dysregulated RAS signaling in pancreatic cancer. However, the RAS/RAL effector pathway has been refractory to inhibition by pharmacologic means.

Our previous studies showed that cyclin-dependent kinase 5 (CDK5) is important for RAL activity in pancreatic cancer. CDK5 knockdown, dominant-negative expression, or treatment with the CDK inhibitor dinaciclib (SCH727965; MK-7965) effectively...
Inhibited RAS/RAL activation and resulted in substantial decreases in cell migration and anchorage-independent growth in vitro, and of growth and metastasis of pancreatic cancer xenografts in vivo (16, 17). Simultaneous blocking of CDK5 and the PI3K/AKT or RAF/MEK/ERK signaling pathways resulted in further inhibition of anchorage-independent growth and cell migration (16). This suggested that such a combination, to inhibit RAL and PI3K/AKT or RAF/MEK/ERK, could be an especially effective therapeutic strategy in pancreatic cancer.

In this study, we show that combining the CDK inhibitor dinaciclib with an inhibitor of the PI3K/AKT pathway, the pan-AKT inhibitor MK-2206, is highly effective in a series of murine orthotopic and subcutaneous patient-derived human pancreatic cancer xenograft models. Based on these data, a phase I clinical trial has been initiated to evaluate this combination in human pancreatic cancer.

**Materials and Methods**

**Chemicals and reagents**

Dinaciclib and MK-2206 were provided by Merck and Co. Dinaciclib was dissolved in 20% hydroxypropyl-β-cyclodextrin (HPBCD; Sigma; ref. 18). MK-2206 was dissolved in 0.5% methanol, 0.1% Tween-80. The chemical structures of dinaciclib and MK-2206 have been published previously (19, 20).

**Generation of orthotopic and subcutaneous xenografts and drug treatment**

All small animal experiments described conformed to the guidelines of the Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD). Mice were maintained in accordance with the guidelines of the American Association of Laboratory Animal Care.

**Orthotopic xenograft studies**. Two modestly gemcitabine sensitive, patient-derived pancreatic cancer xenograft models, Panc265 and Panc253, were chosen to examine the effect of dinaciclib, MK-2206, and dinaciclib + MK-2206 in inhibiting tumor growth and metastases of pancreatic cancer. Low-passage subcutaneous xenograft tissue was minced and implanted orthotopically in the pancreas of athymic nude mice, as described previously (21). Mice were measured by ultrasound (Vevo660, VisualSonics) and randomized by tumor size into 4 treatment groups (n = 7 per group: vehicle control, dinaciclib, MK-2206, and dinaciclib + MK-2206) immediately preceding initiation of therapy (days 14–45 after implantation). The coefficient of variance among tumor volumes at the start of treatment was ≤10% in each experiment. Treatments were as described above. The total body weights were measured weekly. At the end of the treatment, the mice were euthanized. The primary tumors were harvested and weighed, and the tumor volume was measured with calipers of three orthogonal diameters (a, b, and c) and calculated using the formula volume = 1/2(abc). Spleen, pancreas, liver, intestine, colon, lymph node, pituitary, diaphragm, kidney, and lungs were inspected by a thorough necropsy for grossly visible metastases. Because direct, low-passage patient-derived xenograft (PDX) models were used throughout this study, fluorescent or luminescent assays were not applicable; visual inspection has proven to be accurate for the evaluation of metastases in our PDX models (21–24).

**Subcutaneous xenograft studies**. Subcutaneous murine xenografts of low-passage patient-derived human pancreatic cancers were generated as previously described (16, 17, 25). Six PDX models were chosen at random from the Johns Hopkins PDAC BioBank (25). In each model, tumors were transplanted into both flanks of 20 male CD1 nu/nu athymic mice each. Three to 8 weeks after subcutaneous implantation, mice for each case were randomized into four groups of five mice each, and assigned to receive treatment with vehicle control, dinaciclib, MK-2206, or dinaciclib + MK-2206. Total body weights were determined weekly, and xenograft tumor volumes were measured weekly using a digital caliper as previously described (17). After 3 to 5 weeks of treatment, mice were euthanized and tumor tissues were harvested. Tissue samples were preserved in formalin solution for histology and immunohistochemistry.

**Immunohistochemistry**

For Ki67, cleaved caspase-3, and p-AKT staining of formalin-fixed paraffin-embedded tissue sections, anti-Ki67, or cleaved caspase-3, and p-AKT primary antibody (clone K2; Ventana Medical Systems) was used in combination with a Ventana Benchmark Autostainer as described (16, 17). Phospho-Rb (Ser807/811) was stained using a rabbit anti-human polyclonal antibody (#9308, Cell Signaling Technology) at a dilution of 1:300 following the standard recommendations provided by the manufacturer. To determine the positive staining cells for Ki67 or the difference in staining intensity for caspase-3, p-Akt, and pRb, 10 different but histologically similar fields were selected per sample, and the slides were analyzed quantitatively using NIH ImageJ software (26). The staining intensity or positive cells measured by the software was plotted using Graph Pad 6.01 (GraphPad Software, Inc.).

**Statistical analysis**

Two-tailed t-tests were performed using Prism version 6.01. P < 0.05 was regarded to be statistically significant. Bar diagrams were generated using Prism version 6.01 and show means and SEMs if not otherwise indicated.

**Results**

Effective combined treatment with dinaciclib and MK-2206 in orthotopic and subcutaneous PDX models of PDAC

Our previous results showed that CDK5 activity is critical for the RAS/RAL signal transduction pathway in PDAC (16), and that the CDK inhibitor dinaciclib inhibits orthotopic PDAC xenograft growth (17). It has been shown in preclinical studies that inhibition of multiple RAS effector pathways, especially combinations including RAL (27), can be especially effective in limiting growth of RAS-driven human cancers. Therefore, in the current set of experiments, we examined the effect of inhibiting CDK/RAL in combination with another central RAS effector pathway, the PI3K/AKT pathway. We initially chose two highly characterized PDX models of PDAC, Panc253 and Panc265, from the Johns Hopkins PDAC BioBank (25). These KRAS-mutant models closely resemble the pathologic conditions of pancreatic cancer in humans. A 2- to 3-mm3 tumor explant was implanted into the pancreas of nude mice, and ultrasound imaging was used to measure the tumor size before randomization and treatment, which began when tumors grew to 50 to 100 mm3. The combination of dinaciclib (20 mg/kg, i.p., three times a week) and MK-2206 (60 mg/kg, orally, three
times a week) was well tolerated by the mice, and dramatically blocked tumor growth in the orthotopic Panc265 (90.0%, \( P < 0.001 \)) and Panc253 (93.0%, \( P < 0.001 \)) models (Fig. 1). It also markedly reduced the number of metastatic lesions in both Panc265 (88.2%, \( P < 0.001 \)) and Panc253 (99.0%, \( P < 0.001 \)) tumor models (Fig. 1; Supplementary Fig. S1). Remarkably, complete responses were induced by the combination treatment of dinaciclib and MK-2206 in one mouse in the Panc265 tumor model, and in two mice in the Panc253 tumor model.

We then examined a larger panel of PDAC PDX models for their sensitivity to the combination of dinaciclib and MK-2206. These six additional models were chosen at random from the Johns Hopkins PDAC BioBank, and examined as subcutaneous xenografts. All of these models have KRAS mutations. The results, shown in Fig. 2, confirm the efficacy of the combination dinaciclib + MK-2206 treatment. Growth of the tumors was significantly reduced in all six models, including several instances of significant tumor regression. Mean growth inhibition in these PDAC models ranged from 63% to 86%.

**Pharmacodynamic and functional markers**

In order to assess the efficacy of dinaciclib and MK-2206 in inhibiting their targets in the PDAC models, we treated the mice 2 hours before euthanasia and tumor harvest (Fig. 3 and

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**Figure 1.**

Effect of dinaciclib and MK-2206 on orthotopic PDX models. Left, Panc265. Right, Panc253. Tumors were orthotopically implanted in nude mice and allowed to grow to an initial mean size of 70 ± 16 mm³ or 74 ± 12 mm³ (mean ± SE) in Panc265 and Panc253, respectively, measured by ultrasound, at which point the treatment was initiated. Treatment groups included vehicle control, dinaciclib (20 mg/kg three times a week), MK-2206 (60 mg/kg three times a week), and dinaciclib + MK-2206. Treatment continued for 25 days, after which the tumors were harvested and weighed, and metastases were counted. A, primary tumor volume. Combined treatment of dinaciclib and MK-2206 significantly impaired the growth of the primary tumors. B, metastases were quantitated as described in Materials and Methods. Combined treatment of dinaciclib and MK-2206 markedly inhibited the metastases of orthotopic patient-derived pancreatic cancer xenografts. C, primary tumors. Note complete responses. D, body weight of mice during treatment.
Supplementary Fig. S2). For dinaciclib, we examined phospho-RB (pS807/811), a well-characterized target of several CDKs (28), and for MK-2206, we examined phospho-AKT (pS473), by Western blotting. Both dinaciclib and MK-2206 efficiently inhibited their respective targets (Fig. 3A and Supplementary Fig. S2). We also examined the ability of dinaciclib and MK-2206 to inhibit cell proliferation, as assessed by immunohistochemistry for Ki67, and to induce apoptosis, as assessed by immunohistochemistry for cleaved caspase-3. Figure 3B and Supplementary Fig. S2 show that either dinaciclib or MK-2206 had a moderate effect in reducing cell proliferation, and that this effect was markedly augmented by combination dinaciclib + MK-2206 treatment. Similarly, apoptosis was induced by each compound, and the combination resulted in increased apoptosis. These results, demonstrating both inhibition of cell proliferation and induction of apoptosis, are consistent with the tumor growth inhibition results shown earlier (Figs. 1 and 2).

Discussion

In this study, we explored the effect of a novel combination to target two major effector pathways of RAS signaling, RAL and PI3K, in PDX models of pancreatic cancer. We had shown previously that dinaciclib blocked RAL activation in pancreatic cancer.
Figure 3.
Both dinaciclib and MK-2206 are active in xenografts, inhibit proliferation, and induce apoptosis. Subcutaneous xenografts from models PC354 and PC374 were harvested 2 hours after the final treatment and were examined by immunohistochemistry. A, pharmacodynamic biomarkers. Rb phosphorylation, a marker of CDK activity, was inhibited by dinaciclib, but not MK-2206, treatment. AKT phosphorylation was inhibited by MK-2206, but not dinaciclib, treatment. B, growth and viability. Cell proliferation (Ki67) was inhibited, and apoptosis (cleaved caspase-3) was induced, by both dinaciclib and MK-2206. Quantitation of immunohistochemistry for each marker is shown on the right.
cells and inhibited human pancreatic cancer xenograft growth in mice (17). Our current study was based on our earlier data indicating that simultaneous inhibition of CDK5 and the PI3K pathway resulted in substantially more effective inhibition of anchorage-independent growth of PDAC cells (16). As these earlier studies suggested, the combination of dinaciclib and MK-2206 was quite effective in our PDX models.

Dinaciclib is a potent inhibitor of CDK5 (19), and, in turn, RAL (16, 17). Our previous studies showed that CDK5 is a relevant target in PDAC, at least in part through its role in RAL activation (16). However, our studies do not indicate that the sole relevant target of dinaciclib is CDK5/RAL. Like other reported ATP-competitive CDK5 inhibitors (29), dinaciclib inhibits several other CDKs, presumably due in large measure to the homology among the ATP binding pockets of members of the CDK family. Thus, dinaciclib is also a very efficient inhibitor of CDKs 1, 2, and 9 (19); these CDKs have been shown to be significant targets for cancer therapy (30) and may contribute to the antitumor effects we have shown.

Moreover, CDK5 has targets, in addition to the RAS/RAL pathway, that are relevant to cancer biology (31–33). CDK5 was originally characterized as a neuronal protein involved in cell migration, adhesion, and cytoskeletal remodeling (34). These processes are important in cancer progression, and CDK5 has also been shown to be important for these processes in cancer cells, in a cell-specific manner, through phosphorylation of target proteins, including FAK, Talin, RAPGEF2, and PIKE-A (35–40). CDK5 participates in cell growth and survival, in part by phosphorylation of targets including Rb, NOXA, and STAT3 (41–46). Effects of CDK5 on secretion, inflammation, and angiogenesis are also well characterized (32, 33). These targets likely contribute to the overall effect of CDK5 inhibition on the reduced xenograft growth we have observed. While the relative contributions of the various targets of dinaciclib or CDK5 will be difficult to dissect, the contribution of the RAS/RAL pathway may be addressable in future studies, because promising preclinical inhibitors of RAS and RAL have been reported recently (10, 47–52).

MK-2206 is an allosteric pan-AKT inhibitor with high specificity for AKT (53). The PI3K/AKT pathway is dysregulated in many cancers and is a well-validated therapeutic target. MK-2206, especially in combination, has been shown to have clinical activity against several malignancies (54, 55, 56), and to sensitize cancer cells to a variety of therapeutic compounds and radiation (57, 58). This sensitization may be due in part to the ability of PI3K/AKT activity to augment a large number of survival pathways, including antiapoptosis, DNA repair, and metabolic processes (58, 59); such mechanisms may contribute to the significantly improved response to the combination of dinaciclib and MK-2206, compared with either compound alone, that we report here.

Based on the promising results of this study, a multicenter phase 1 clinical trial (NCT01783171) of a combination of dinaciclib and MK-2206 for pancreatic cancer has been opened.

Disclosure of Potential Conflicts of Interest

R. Banerjee has ownership interest (including patents) in dinaciclib (SCH 727965 and MK-7965). R. Booher has ownership interest (including patents) in Merck. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Hu, B.D. Nelkin, A. Maitra
Development of methodology: C. Hu, V. Chenna, R. Booher, A. Maitra
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Dadon, S. Yabuchi, R. Banerjee, B.D. Nelkin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Hu, S. Yabuchi, N. Azad, B.D. Nelkin, A. Maitra
Writing, review, and/or revision of the manuscript: C. Hu, V. Chenna, R. Banerjee, P. Strack, N. Azad, B.D. Nelkin, A. Maitra
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Hu, V. Chenna
Study supervision: B.D. Nelkin

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