Attenuation of the Rb pathway in Pancreatic Neuroendocrine Tumors Due to Increased Cdk4/Cdk6

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**Purpose:** In mice, genetic changes that inactivate the retinoblastoma (Rb) tumor suppressor pathway often result in pancreatic neuroendocrine tumors (Pan-NETs). Conversely in humans with this disease, mutations in genes of the Rb pathway have rarely been detected, even in genome-wide sequencing studies. In this study, we took a closer look at the role of the Rb pathway in human Pan-NETs.

**Experimental Design:** Pan-NET tumors from 92 patients were subjected to immunohistochemical staining for markers of the Rb pathway. To search for amplifications of Rb pathway genes, genomic DNAs from 26 tumors were subjected to copy number analysis. Finally a small molecule activator of the Rb pathway was tested for effects on the growth of two Pan-NET cell lines.

**Results:** A majority of tumors expressed high amounts of Cdk4 or its partner protein Cyclin D1. High amounts of phosphorylated Rb1 were present in tumors that expressed high levels of Cdk4 or Cyclin D1. The copy numbers of Cdk4 or the analogous kinase gene Cdk6 were increased in 19% of the tumors. Growth of the human Pan-NET cell line QGP1 was inhibited in a xenograft mouse model by the Cdk4/6 inhibitor PD 0332991, which re-activates the Rb pathway.

**Conclusions:** Inactivation of the Rb pathway was indicated for most Pan-NETs. Gene amplification and overexpression of Cdk4 and Cdk6 suggests that patients with Pan-NETs may respond strongly to Cdk4/6 inhibitors that are entering clinical trials.
Introduction

Pancreatic neuroendocrine tumors (Pan-NETs) represent the second most common epithelial neoplasm in the pancreas, accounting for 1-2% of pancreatic tumors (1). Originally considered to be a benign group of insulin-producing tumors (insulinoma), it has now become apparent that 50% or more Pan-NETs are non-functional, and help to comprise a heterogeneous group of tumors with often unpredictable and varying degrees of malignancy (2). Pan-NETs pose significant challenges in clinical management. Up to 50% of patients have liver metastases at the time of initial diagnosis, and median survival for patients with liver metastasis is 24 months (3). Currently, there are no reliable biomarkers to predict the recurrence and progression of the disease.

Although Pan-NETs may respond to streptozotocin or temozolomide, the majority of low and intermediate grade tumors are insensitive to standard cytotoxic chemotherapy. This has led to efforts to explore pathways affected by oncogenes and tumor suppressors in these tumors, in hopes of predicting better treatment strategies. Mutations associated with pancreatic neuroendocrine tumorigenesis occur in genes involved in chromatin remodeling, such as MEN1, ATRX, and DAXX (4, 5), but these associations have not yet led to treatments. On the other hand, patients with tuberous sclerosis occasionally present with Pan-NETs (6-8), and the fact that mutations causing tuberous sclerosis activate the mTOR protein led to the idea that patients with Pan-NETs would benefit from treatment with the mTOR inhibitor rapamycin. Indeed, the rapamycin analogue everolimus was recently approved as a treatment for this disease (3); moreover, mutations in mTOR pathway genes TSC2 and PTEN have recently been found in 15% of Pan-NETs. At the level of tumor anatomy, Pan-NETs are remarkably vascularized, and the angiogenesis inhibitor sunitinib has also been approved for treatment of patients with Pan-NETs (9).

A critical step in tumorigenesis is entry into S phase of cell cycle in the absence of growth signals. The Rb1 tumor suppressor plays an important role in regulating this step, by preventing the activity of E2F transcription factors that are critical for expression of genes involved in S phase metabolism (Figure 1) (10). Loss of Rb1 function results in unchecked transcriptional activation by elongation factor 2 (E2F) and can occur either by loss of Rb1 protein itself via Rb1 gene mutations or by aberrations in other regulatory elements of the Rb1 pathway that increase phosphorylation of the Rb1 protein (Figure 1). In fact, 80% of cancers maintain an intact Rb1 protein but display genetic alterations of other components of the Rb1 pathway (11). Rb1 is negatively regulated via phosphorylation by cyclin-dependent kinases Cdk4 and Cdk6, which are activated by cyclin D1 and negatively regulated by a number of cyclin-dependent kinase inhibitors. Tumors with an intact Rb1 gene may show chromosomal rearrangement or amplification of Cdk4 or D-cyclin genes, as well as loss or repression of p16Ink4a, which encodes an inhibitor of Cdk4/6 (12-14).
In this study, we investigated abnormalities in the Rb1 pathway in Pan-NETs and identified significant genetic aberrations which culminated in attenuated Rb1 function, and which could be restored by a small molecule, PD 0332991.
Translational Relevance

Neuroendocrine tumors are the second most common neoplasia of the pancreas. We provide evidence that the Rb tumor suppressor pathway is inactivated in a high majority of Pan-NETs. We demonstrate that one of the mechanisms by which Rb pathway inactivation occurs is through amplification of genes encoding the cyclin-dependent kinases Cdk4 or Cdk6, which we detect in 19% of patients. We also show that in Pan-NET cell lines Cdk4 expression decreases upon treatment with low doses of rapamycin, suggesting that mutations that activate the mTOR pathway, which are found in 15% of Pan-NETs, may also increase expression of Cdk4. An inhibitor of Cdk4/6, PD 0332991, blocks growth of two human Pan-NET cell lines, and acts in synergy with rapamycin. Our data suggest that CDK4/6 inhibitors could prove beneficial to patients with Pan-NETs, either alone or in combination with analogues of rapamycin.
Materials and Methods

Patients and clinical data. Cases of well-differentiated pancreatic neuroendocrine tumors, including both functioning and non-functioning tumors, and corresponding clinical data were collected from institution databases from 1996-2008. The study was approved by MSKCC's Internal Review Board. Disease-related factors included the date and location of recurrence, and survival as measured to the time of last follow-up or death. Patient status at last follow-up was documented as no evidence of disease, alive with disease, dead of disease, and dead of other causes. Clinicopathologic variables were assessed for their association with recurrence and survival. Recurrence-free survival (RFS) and disease-specific survival (DSS) were calculated using the Kaplan-Meier method, and comparisons were made using the log-rank test. Significance was defined as $p < .05$.

Tissue samples. Formalin-fixed and paraffin-embedded (FFPE) tissue blocks (n=92) were used for tissue microarray (TMA) construction for IHC staining and also for fluorescence in situ hybridization (see below). Fresh frozen tissue was available for gene expression assay by quantitative real time PCR on 41 cases, 36 of which were also within the FFPE tissue microarray.

Cell lines. The pancreatic neuroendocrine tumor cell line QGP1 was purchased from the Japan Health Sciences Foundation. BON1 was a gift from the lab of Kjell Oberg. MCF7 and SAOS2 were purchased from ATCC. Cell lines were grown at $37^\circ$C under 5% CO$_2$. All lines were grown in DMEM supplemented with 10% fetal bovine serum except QGP1, which was grown in RPMI supplemented with 10% fetal bovine serum. DMEM and RPMI were purchased from Invitrogen. Fetal bovine serum was purchased from Sigma-Aldrich.

Construction of tissue microarrays. Hematoxylin and eosin stained slides of the pancreatic resection specimens were reviewed by one pathologist, and slides containing tumor were marked and matched with corresponding paraffin blocks. Tissue cores of 0.6 mm were then punched out in triplicate from locations randomly selected within the marked tumor areas and mounted in blank recipient blocks using an automated tissue microarrayer (Beecher Instruments, Inc.).

Immunohistochemistry (IHC): IHC staining for Cdk4, phospho-Rb1, and cyclin D1 were performed on tissue microarray (TMA) from representative paraffin blocks with tumor tissues in triplicate cores for each case. The sections were de-paraffinized in xylene, rehydrated with ethanol, then steamed for 30 minutes with citrate buffer (0.01 M citric acid, pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked using 95 ml of methanol plus 5 ml of 3% hydrogen peroxide solution. Non-specific protein binding was blocked with 1% BSA for 60 minutes. Rabbit anti-phospho-Rb1 antibody (Ser807/811), rabbit monoclonal Cyclin D1 and mouse monoclonal Cdk4 were from Cell Signaling Technology, Neo...
Markers and Invitrogen, respectively. Positive controls were included with each antibody and negative controls were obtained by omitting the primary antibodies. Correlation between protein expression was determined using Pearson’s Product-moment coefficient (R) with p values.

**DNA copy number assays.** Genomic DNA was isolated from either human tumors or cell lines using a Wizard kit from Promega. Copy number assays for Cdk4, Cdk6, Cdk2, or Gli1 were performed by real time PCR using an Applied Biosystems Prism 7500. TERT and RNaseP were used to normalize the quantitation, and only tumors that showed amplification relative to both TERT and RNaseP were called amplified. All copy number assays were purchased from Applied Biosystems.

**Quantitative real time – polymerase chain reaction (qRT-PCR)** RNA was extracted from fresh frozen tissue using Qiagen RNEasy Mini Kit® (Qiagen), then reversely transcribed into cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems). Cdk4 and Cdk6 transcription was evaluated using TaqMan® Real Time PCR Gene Expression Assays (Applied Biosystems). The reaction was carried out on the Applied Biosystems 7500 Fast Real Time PCR System using TaqMan® Master Mix. A house keeping mRNA, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) was assessed in all samples. Paired normal pancreatic tissue was used as control (transcript = 1), and sample transcript was calculated against HGPRT and normal control and expressed as $2^{-\Delta\Delta Ct}$. p values were determined by Fisher’s exact test.

**Fluorescence in situ hybridization (FISH).** The Cdk4 probe contained BAC clones RP11-571M6 and RP11-970A5 labeled with Red dUTP, and the chromosome 12 centromere reference probe was plasmid clone pα12H8, labeled with Green dUTP (dUTPs from Enzo Life Sciences, Inc., supplied by Abbott Molecular Inc.). Probes were labeled by nick translation and hybridized to tissue sections according to standard procedures. RP11-571M6 was part of a 1Mb clone set kindly provided the Wellcome Trust Sanger Institute (15). BAC clone RP11-970A5 was purchased from BACPAC Resources. Briefly, paraffin sections of the tissue microarray were de-paraffinized in xylene, treated with 10 mmol/L sodium citrate buffer (pH 6.0) then with pepsin-HCl. The probe mixture was applied to the slides, denatured on a HYBrite automated hybridizer (Vysis, Abbott Molecular), then incubated overnight at 37°C. After hybridization, the slides were stained with 4′,6-diamidino-2-phenylindole and mounted in antifade (Vectashield, Vector Laboratories). Samples were analyzed using an automated imaging system (MetaSystems) and Isis 5.0 scanning and imaging software. A minimum of 100 cancer cells were evaluated for each case, whenever possible.

**Cell growth and arrest assays.** For in vitro studies, PD 0332991 was purchased from Selleck Chemicals and rapamycin was purchased from Sigma Aldrich. For measurement of IC50, drug was re-added daily.
Cell number was measured in quadruplicate wells by assay of ATP using a CellTiter-Glo kit (Promega). Cell number was assayed prior to drug addition, and again after four days of drug treatment or mock treatment. For measurement of effects on cell cycle, drug was added for three hours (Figure 5D) or seven hours (Figure 6D), then media was removed and drug was re-added along with 100 ng/ml nocodazole (Sigma Aldrich) for 16 hours. Cells were harvested by trypsinization 16 hours after addition of nocodazole, washed in PBS, and fixed in 70% ethanol. Propidium iodide was then added and cell cycle analysis was performed using a FACScaliber instrument (BD Biosciences). The Bliss additivity value was computed as the sum of the percentage G1 arrest caused by either drug alone, minus the product of both of the percentage G1 arrest values.

**Immunoblots.** Antisera raised against Cdk4, Rb1 or Rb1 phosphorylated at Serine 780 were purchased from Novus Biologicals. β-actin antisera were purchased from Sigma Aldrich. To determine steady state expression of Cdk4, NET cell lines QGP1, BON1 or H727 were plated for 36 hours, then protein was extracted using radioimmunoprecipitation assay buffer. To measure the effect of PD 0332991 on Rb1 phosphorylation, BON1 or QGP1 cells were plated for 24 hours and drug was then added for seven hours, followed by extraction of protein. To determine the effect of rapamycin on Cdk4 expression, cells were plated for 24 hours and drug was then added for 14 hours followed by extraction of protein. Protein was separated by SDS-polyacrylamide gel electrophoresis using NuPage 4%-12% Bis-Tris gels (Invitrogen), then transferred to a polyvinylidene fluoride filter for Western blotting.

**Xenograft studies.** Mouse studies were performed under the guidelines and approval of the Rutgers University Institutional Animal Care and Use Committee. Female nude mice were purchased from Jackson Laboratories. Five million QGP1 cells were suspended in 0.5 ml of phosphate-buffered saline, and injected subcutaneously into the flank of each mouse. Drug treatment began two weeks later. PD 0332991, purchased from Chemietek, was dissolved in PBS at a concentration of 3 mg/ml. Mice were weighed before treatment and then provided either with PBS alone, or with 150 mg/kg of drug by oral gavage. Mice were treated for five consecutive days, then given a two day respite, then re-treated for another five days. Tumors were measured by caliper on days 0, 6, 8, 10, 12, and 14. Tumor volume was computed as length multiplied by the square of the width. Six mice were in the control and six mice were in the PD 0332991 arm of the study. One mouse in the PD 0332991-treated group died on day 10.
Results

Pan-NETs express high levels of Cdk4 and its product, phospho-Rb1

Immunohistochemistry was carried out on a tissue microarray constructed from 92 cases of well-differentiated Pan-NETs. Clinical characteristics of this database were previously published (16, 17). Representative cases are shown in figure 2. Although neither Cdk4 nor phospho-Rb1 could be detected in normal islets (data not shown), Cdk4 staining was detected in 58% of Pan-NETs and phospho-Rb1 was detected in 68% of tumors. Phospho-Rb1 was found either exclusively in the nucleus, or else in both nucleus and cytoplasm. There was a statistically significant correlation between phospho-Rb1 and Cdk4 protein expression (r=0.55; p=0.01). Another kinase of Rb1, Cdk6, was not tested due to poor antisera, but we did look at Cyclin D1 expression. Cyclin D1 staining occurred in 68% of Pan-NETs but not in normal islets. Like Cdk4, Cyclin D1 expression correlated well with Rb1 phosphorylation (r=0.51; p=0.03). High expression of Cyclin D1 has previously been reported in Pan-NETs (18, 19), but high amounts of Cdk4, phospho-Rb1, and correlation analyses are novel results. We found no significant correlation of either Cdk4 or high phospho-Rb1 with either recurrence or disease free survival with a mean clinical follow up of 55 months (p>0.05).

Quantitative RT-PCR was performed using mRNA from fresh frozen samples of well-differentiated Pan-NETs. When compared with normal pancreas tissue, the Cdk4 transcript was markedly increased ranging from 1.2 to 97 fold (mean 12.5±2.5) (Figure 3A). In contrast, increased Cdk6 transcript was less common with mean transcription levels of 0.8±0.1 when compared to control normal tissue (Figure 3B). Interestingly, Cdk4 transcripts were significantly higher in non-functional Pan-NETs (mean 14.6±3.0) when compared with functional Pan-NETs (mean 3.8±0.5), which were represented by clinically symptomatic insulinoma, glucagonoma, and vasoactive intestinal peptide (VIP) producing tumor (VIPoma) (Figure 3C).

Although recent exomic sequencing of Pan-NETs did not reveal point mutations in Rb pathway genes (4), our observations of high expression of Cdk4 RNA and protein, along with high Rb1 phosphorylation, indicated the possibility of amplification of the Cdk4 gene in these tumors. Indeed, real time PCR analysis revealed Cdk4 or Cdk6 to be amplified in a subset of tumors (Figure 4A and 4B). Out of 26 tumors tested, three had a higher dosage of the Cdk4 gene, and two had a higher dosage of the Cdk6 gene. Consistent with the overlapping functions of the two genes, tumors with amplified Cdk4 did not have amplified Cdk6, although this observation was not statistically significant due to the small sample size. Altogether 19 percent of tumors showed amplification of either Cdk4 or Cdk6. The level of amplification was small, in the range of 1-2 additional copies (figure 4A and 4B). We also used real time PCR as a method to test for amplification of genes proximal to Cdk4, and saw no examples within this 26 patient data set of increased copy number of the Gli1 or Cdk2 genes, which reside within 300 and 1800
kilobases, respectively, on either side of the Cdk4 gene on chromosome 12 (data not shown). Therefore, amplification of Cdk4 appears to be specific to this gene locus. Cyclin D1, which resides on chromosome 11, was not amplified within this data set (data not shown).

We further investigated Cdk4 gene status by fluorescence in situ hybridization (FISH) of the 92 cases within our tissue microarray, and observed tumors in which Cdk4 was present in 3 or 4 copies but in which there were only two copies of the centromere of chromosome 12 (Figure 4C). These tumors therefore appear to have extra copies of Cdk4 due to amplification, and not due to G2 arrest or polysomy.

**Neuroendocrine cell lines and Cdk4/6**

We examined pancreatic neuroendocrine cell lines BON1 and QGP1 and pulmonary neuroendocrine cell line H727 in hopes of finding an in vitro model system for Cdk4 amplification/expression in human NETs. While BON1 did not show amplification of Cdk4, the QGP1 and H727 cell lines had 6 and 3 copies of the Cdk4 gene, respectively (Figure 4A). Gene copy number correlated with Cdk4 mRNA and protein expression levels in these three cell lines as assessed by quantitative RT-PCR and Western blot, respectively (Figure 5A). There was an extra copy of Cdk6 in QGP1, but this gene was not amplified in H727 or BON1 (Figure 4B).

The pharmaceutical compound PD 0332991 selectively inhibits the activity of both Cdk4 and Cdk6 (20). Growth of the two pancreatic neuroendocrine cell lines, QGP1 and BON1, responded in a dose-dependent fashion to treatment with PD 0332991 (Figure 5B). Interestingly QGP1, the cell line with amplified Cdk4, was particularly sensitive to PD 0332991, with an IC50 of only 36 nM. BON1, which expresses less Cdk4 and is not amplified for the gene, had a higher IC50 of 155 nM. As shown in figure 5C, treatment of QGP1 and BON1 cells with PD 0332991 for only seven hours significantly decreased phospho-Rb1 levels without changing the total amount of Rb1 protein. The human breast cell line MCF7 was previously reported to be a strong responder to PD 0332991 (21), and we measured an IC50 value of 125 nM for this line (data not shown), which compares favorably with the IC50s of QGP1 and BON1. We also measured a very high IC50 (>1000 nM; data not shown) for the osteosarcoma cell line SAOS2. This is consistent with a known deletion of the Rb1 gene in SAOS2, which renders its growth less dependent on Cdk4 activity.

If PD 0332991 inhibits cell line growth by targeting Cdk4/6, then it should arrest growth at the G1 phase of the cell cycle. Indeed, a very large percentage of BON1 and QGP1 cells remained in G1 phase upon PD 0332991 treatment (Figure 5D). In this experiment, growth arrest of cells treated with nocodazole alone, which blocks in G2, were compared with cells pre-treated with PD 0332991 for three hours, then co-
treated with PD 0332991 and nocodazole. Pretreatment with PD 0332991 resulted in a 10-fold and 6-
fold increase in G1 arrest for QGP1 and BON1, respectively. These data show that low concentrations of
PD 0332991 can block Cdk4 activity in two pancreatic NET cell lines, reactivate Rb1, and halt cell growth
at G1.

Next we tested the effect of PD 0332991 on growth of the QGP1 cell line in vivo. QGP1 cells were
injected sub-cutaneously into the flanks of nude mice in order to establish xenografted tumors. After
two weeks, the mice were treated either with phosphate-buffered saline or with 150 mg/kg of PD
0332991. As shown in figure 6A, the tumors within the PBS-treated group became very large, whereas
the tumors within the PD 0332991-treated group did not grow. The difference in tumor volume
between the two groups was statistically significant by day 10, and remained statistically significant at
days 12 and 14.

Finally, we investigated the effect of rapamycin upon Cdk4 expression and upon the activity of PD
0332991. A previous study showed that rapamycin treatment can decrease expression of Cdk4 by other
cell lines (22), and in figures 6B and 6C we show that this is also true for BON1 and QGP1. The dose of
rapamycin required to lower Cdk4 is much less than the IC50 of either cell line, which we determined to
be 1 nM and 3 nM for BON1 and QGP1, respectively (data not shown). We wondered whether
rapamycin and PD 0332991 might be able to function in combination to block the cell cycle: rapamycin,
by lowering Cdk4 expression and PD 0332991, by inhibiting the Cdk4 that remained. As shown in figure
6D, a sub-IC50 dose of rapamycin alone, or PD 0332991 alone, resulted in very small increases in the
population of cells in G1. However, combined low doses of the two drugs increased G1 arrest. The
observed G1 arrest caused by the combination of drugs is much higher than the Bliss additivity value,
which estimates the effect of two drugs if they act independently. Therefore in either cell line,
rapamycin decreases Cdk4 expression and acts synergistically with PD 0332991.
Discussion

In this study, we have shown that high expression of Cdk4 coincides with Rb1 phosphorylation in a majority of pancreatic neuroendocrine tumors. High Cdk4 expression is due at least in part to increased transcription, as we find higher levels of Cdk4 mRNA in Pan-NETs when compared to normal pancreatic tissue. One mechanism for increased transcription may be amplification of the Cdk4 gene. In 12 percent of the samples tested, the copy number of Cdk4 increased. But amplification alone does not account for all of the tumors in which we observed high Cdk4 expression (12% amplification vs. 58% overexpression). In two Pan-NE cell lines, we show that Cdk4 expression decreases upon inhibiting mTOR with rapamycin; thus, an alternative mechanism for increased Cdk4 expression may be through activation of the mTOR pathway, which occurs by mutation of PTEN and TSC2 in patients with Pan-NETs.

There are other ways to inactivate the Rb pathway in Pan-NETs. For instance, hypermethylation of the promoter for the cyclin dependent kinase inhibitor gene p16\(^{INK4a}\) has been reported to occur in Pan-NETs (23). Also, we observe increased copy number of Cdk6 in 8% of the samples. Thus about one in five Pan-NETs have higher dosage of Cdk4 or Cdk6. In all cases of amplification of both Cdk4 and Cdk6, the increased gene dosage was a modest 1-2 copies.

Amplification of the Cdk4 gene has previously been reported for a number of tumor types, including breast, melanoma, lymphoma, sarcoma and glioma (24). The involvement of the Cdk4/6-cyclin D1 complex in tumorigenesis of mantle cell lymphoma is particularly well-established due to the presence of a t(11;14)(q13;q32) translocation that puts Cyclin D1 under control of an immunoglobulin enhancer in a high majority of these patients (25). Interestingly, low copy number (<5) increases in dosage of the Cdk4 gene, without co-amplification of a nearby gene on chromosome 12q13, have been found in the more aggressive blastic form of these tumors (26). As is true for mantle cell lymphoma, we have also found high expression of Cyclin D1 as well as low copy number, locus-specific increases in the dosage of the Cdk4 gene. Given this similarity, it is perhaps notable that in a small trial, a subset of patients with relapsed mantle cell lymphoma responded well to the Cdk4 inhibitor PD 0332991 (27).

Previous experiments in mice have linked the Rb pathway, particularly Cdk4, to pancreatic neuroendocrine tumorigenesis. The RIP1-Tag2 mouse develops insulinomas due to insulin promoter-driven expression of the SV40 T antigen, which inactivates both the Rb and p53 families of tumor suppressor genes (28). Mice doubly knocked out for the p16\(^{INK4c}\) and p27\(^{KIP1}\) cyclin dependent kinase inhibitor genes develop islet cell hyperplasia (29). Cdk4(R24C) mice express an allele of Cdk4 that cannot be downregulated by p16\(^{INK4a}\), and these mice produce a large variety of tumors, including pancreatic neuroendocrine tumors (30, 31). Indeed, Cdk4 has been called a “driver” of Rb pathway-
dependent tumorigenesis due to the high penetrance of tumor formation in Cdk4(R24C) mice as compared to much weaker cancer phenotypes for mice with mutations in other genes of the Rb pathway (32). In a complementary experiment, mice lacking Cdk4 were shown to be relatively healthy with the notable exception of diabetes, due to depletion of pancreatic beta cells (30). More recently it was shown that Cdk4 acts at a very early stage in islet cell development by stimulating the replication of neuroendocrine stem cells (33).

The present study may be of significant clinical value. PD 0332991 is currently in Phase II trials for other human malignancies, and another Cdk4/6 inhibitor is in a phase I trial (http://clinicaltrials.gov/ct2/show/NCT01237236; http://clinicaltrials.gov/ct2/show/NCT01037790). Tumors in which Cdk4 or Cdk6 are overexpressed may respond particularly well to treatment with PD 0332991 as does the QGP1 cell line, which has a Cdk4 amplification and high sensitivity to PD 0332991.

Importantly, PD 0332991 shows synergy with rapamycin in vitro. Rapamycin treatment can result in side-effects including infections due to immunosuppression, and it is possible that combined treatment with PD 0332991 may lower the required dose of rapamycin analogues and thereby reduce its side effects. Interestingly, the BON1 cell line, which has lower expression of Cdk4 than QGP1 but high sensitivity to rapamycin, was particularly sensitive to combination treatment with PD 0332991 and rapamycin. Thus the efficacy of Cdk4/6 inhibition may not be strictly limited to patients with Cdk4 overexpression and/or gene amplification.
Acknowledgments

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References


FIGURE LEGENDS

Figure 1. The Rb tumor suppressor pathway. In the absence of growth signals, tumor suppressor protein Rb1 prevents cells from entering the S phase of cell cycle via inhibition of E2F transcription factors. If Rb1 is phosphorylated by cyclin-dependent kinases, E2F factors are no longer inhibited and activate transcription of genes required for DNA replication and S phase metabolism. Cyclin-dependent kinases Cdk4 or Cdk6, when complexed with cyclin D1, phosphorylate Rb1. In turn, cyclin-dependent kinase inhibitors such as p16INK4a, p18INK4c, and p27KIP1 can inhibit the activity of Cdk4 or Cdk6 complexes. PD 0332991 is a small molecule that selectively inhibits Cdk4- and Cdk6-dependent phosphorylation of Rb1.

Figure 2. Immunohistochemistry of proteins involved in Rb1 phosphorylation. Representative tumors expressing Cdk4, phosphorylated Rb1, and cyclin D1 are shown. Out of 92 cases, immunoreactivity of Cdk4, phosphorylated Rb1, and cyclin D1 was detected in 58%, 68%, and 68% of Pan-NETs, respectively.

Figure 3. Expression of Cdk4 and Cdk6 Transcripts in Pan-NETs. (A) and (B) Relative amounts of Cdk4 or Cdk6 mRNA in Pan-NET samples in comparison to transcription by normal islets of the pancreas, whose level was arbitrarily set at 1. All mRNAs were quantified by real time RTPCR and normalized to hypoxanthine-guanine phosphoribosyltransferase (HGPRT). (C) The same samples as in figure 3A are binned into nonfunctional vs. functional neuroendocrine tumor type. These two groups show statistically significant differences (p < 0.001) as determined by Fisher’s exact test.

Figure 4. Cdk4 and Cdk6 gene amplifications in clinical samples and cell lines. (A) and (B). Copy number assays for Cdk4 and Cdk6 using DNA samples from 26 different human tumor samples, each of which is from a separate patient. NB was obtained from blood from a mixed population of normal donors. B, Q, and H are from human neuroendocrine cell lines BON1, QGP1 and H727, respectively. In these figures, Cdk4 and Cdk6 were normalized to the copy number of TERT within the same sample. (C). Fluorescent in situ hybridization was used to visualize Cdk4 or the centromere of chromosome 12, upon which Cdk4 resides. Cells in this nonfunctioning tumor display four copies of Cdk4 but only two copies of the centromere.

Figure 5. Cdk4 and Rb1 phosphorylation in neuroendocrine cell lines. (A) Protein was extracted from pancreatic NET cell lines QGP1 and BON1, and bronchial NET cell line H727, separated by gel electrophoresis and immunoblotted for Cdk4 and β-actin. Relative levels of Cdk4 mRNA for each cell line are also shown and were measured by real time RTPCR and normalized to β-actin. Cdk4 copy number for each cell line is also shown, as determined in Figure 4A. (B) QGP1 or BON1 were treated with varying concentrations of PD 0332991 for four days, and the amount of cell growth was then measured by assay of intracellular ATP as described in Materials and Methods. (C) QGP1 and BON-1 cells were treated...
without (mock) or with PD 0332991, respectively, for 7 hours prior to Western blot analysis of phospho-Rb1 at Serine-780 (top panel) and total Rb1 protein (middle panel); β-actin was used as endogenous protein control (lower panel). QGP1 was treated with 100 nM PD 0332991 while BON1 was treated with 200 nM. Rb1 had to be split into two panels due to large expression differences between the two cell lines. (D) Pan-NET cell lines QGP1 and BON1 were grown in the presence or absence of PD 0332991 for three hours followed by co-treatment with PD 0332991 and 100 ng/ml nocodazole for an additional 16 hours. QGP1 was treated with 100 nM PD 0332991 while BON1 was treated with 200 nM. Cells were then fixed and treated with propidium iodide prior to cell cycle analysis. By itself, nocodazole causes a strong G2 arrest (traces in middle panel) but pretreatment with PD 0332991 reveals G1 arrest (traces in right panel).

Figure 6. Effect of PD 0332991 in vivo, or in combination with rapamycin. (A) Tumor xenografts comprised by human QGP1 pancreatic neuroendocrine cells were established for two weeks in nude mice, which were then treated either with phosphate-buffered saline (PBS) or with the Cdk4/6 inhibitor PD 0332991 at a dose of 150 mg/kg. The upper line shows the control group and the lower line shows the drug-treated group. The difference in tumor volume between the control and drug-treated group was statistically significant (p<.03 by two-tailed t test) on days 10, 12 and 14. (B) and (C) BON1 (B) or QGP1 (C) cells were treated for 14 hours with the indicated dose of rapamycin, then protein extracts were prepared and separated by SDS-polyacrylamide gel electrophoresis. The expression of Cdk4 and β-actin was detected by Western blot. (D) BON1 and QGP1 cell lines were treated with 0.2 nM rapamycin alone (RAP), 20 nM PD 0332991 alone (PDO) or both 0.2 nM rapamycin and 20 nM PD 0332991 (R + P). After seven hours, nocodazole was added and cells were grown for another 16 hours to enhance detection of G1 arrest. G1 arrest was measured by flow cytometry. Also shown is the Bliss additivity value, which estimates the level of G1 arrest that would occur by combining the two drugs if they acted independently. For both QGP1 and BON1, the difference between the Bliss value and the actual value of the drugs in combination was statistically significant (p < .01 by two-tailed t test).
Rb tumor suppressor pathway

S phase metabolism including DNA replication

Figure 1
Figure 2

Cdk4  phospho-Rb1  cyclin D1
**Figure 3A**

<table>
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<th>Functional (N=8)</th>
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<td>14.6±3.0 (1.2-97)</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

**Figure 3B**

<table>
<thead>
<tr>
<th>CDK6</th>
<th>Non-functional (N=33)</th>
<th>Functional (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8±0.5 (2.2-6.4)</td>
<td>0.8±0.1 (0-3.7)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3C**
Figure 4C

*Cdk4*

12cen
Figure 5A

Cdk4
β-actin

QGP1 BON1 H727

Cdk4
β-actin

QGP1 BON1 H727

Figure 5B

Relative light units

QGP1

relative light units

BON1

nM PD0332991

nM PD0332991

Figure 5C

phospho-Rb1
total Rb1
β-actin

QGP1 BON1

Figure 5D

mock-treated cells
18 hr nocodazole
21 hr PD 0332991/18 hr nocodazole

QGP1

BON1

mock-treated cells
18 hr nocodazole
21 hr PD 0332991/18 hr nocodazole

Table: cdk4 copy number and Relative Cdk4 mRNA

<table>
<thead>
<tr>
<th></th>
<th>QGP1</th>
<th>BON1</th>
<th>H727</th>
</tr>
</thead>
<tbody>
<tr>
<td>copy number</td>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Relative mRNA</td>
<td>3.7</td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Figure 5B

Figure 5C

Figure 5D
**Figure 6A**

Length of treatment (days)

Tumor volume

PBS

PD 0332991

**Figure 6B**

BON1 cells

Cdk4

β-actin

nM rapamycin

**Figure 6C**

QGP1 cells

Cdk4

β-actin

nM rapamycin

**Figure 6D**

QGP1

BON1

% increase in G1

RAP PDO R+P Bliss RAP PDO R+P Bliss

**
Clinical Cancer Research

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Laura H. Tang, Tanupriya Contractor, Richard Clausen, et al.


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