Protein Kinase D as a Potential Chemotherapeutic Target for Colorectal Cancer

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Abstract

Protein kinase D (PKD) signaling plays a critical role in the regulation of DNA synthesis, proliferation, cell survival, adhesion, invasion/migration, motility, and angiogenesis. To date, relatively little is known about the potential role of PKD in the development and/or progression of human colorectal cancer. We evaluated the expression of different PKD isoforms in colorectal cancer and investigated the antitumor activity of PKD inhibitors against human colorectal cancer. PKD2 was the dominant isoform expressed in human colon cancer cells. PKD3 expression was also observed but PKD1 expression, at both the RNA and protein levels, was not detected. Suppression of PKD using the small molecule inhibitors CRT0066101 and kb-NB142-70 resulted in low micromolar in vitro antiproliferative activity against multiple human colorectal cancer cell lines. Drug treatment was associated with dose-dependent suppression of PKD2 activation. Incubation with CRT0066101 resulted in G2–M phase arrest and induction of apoptosis in human colorectal cancer cells. Further studies showed that CRT0066101 treatment gave rise to a dose-dependent increase in expression of cleaved PARP and activated caspase-3, in addition to inhibition of AKT and ERK signaling, and suppression of NF-κB activity. Transfection of PKD2-targeted siRNAs resulted in similar effects on downstream pathways as observed with small molecule inhibitors. Daily administration of CRT0066101 resulted in significant inhibition of tumor growth in HCT116 xenograft nude mice. Taken together, our studies show that PKD plays a significant role in mediating growth signaling in colorectal cancer and may represent a novel chemotherapeutic target for the treatment of colorectal cancer. Mol Cancer Ther; 13(5); 1130–41. ©2014 AACR.

Introduction

Colorectal cancer is a major public health problem in the United States and globally. In the United States, it is the second leading cause of cancer mortality (1). For 50 years, the main chemotherapeutic treatment was the fluoropyrimidine 5-fluorouracil (5-FU). From 1996 to 2004, 3 new anticancer agents were approved, which include the oral fluoropyrimidine capecitabine, the topoisomerase I inhibitor irinotecan, and the platinum analog oxaliplatin. Since 2004, the U.S. Food and Drug Administration has approved 5 molecular targeting agents, including the anti-EGF receptor antibodies cetuximab and panitumumab, the anti-VEGF inhibitors bevacizumab and ziv-afibercept, and a small molecule inhibitor that targets multiple tyrosine kinases, regorafenib. Significant advances have been made in chemotherapy treatment options for patients with metastatic disease, such that improvements in 2-year survival are now being reported, with median overall survival rates of 21 to 24 months (2–4). Despite these advances, none of the currently available treatment options have impacted on 5-year overall survival, and cellular drug resistance remains a significant obstacle to successful chemotheraphy (5). Thus, identification of novel signaling pathways and targets that mediate the growth and proliferation of colorectal cancer is critically important for discovering and developing novel therapeutic agents with enhanced antitumor activity that overcome drug resistance and improve overall quality of life.

Protein kinase D (PKD) is a subfamily of serine/threonine kinases of the calcium/calmodulin-dependent kinase superfamily, composed of 3 different isoforms, PKD1, PKD2, and PKD3 (6). This signaling pathway functions downstream of protein kinase C (PKC), G protein–coupled receptors, and tyrosine kinase receptors. PKD can be activated in a PKC-dependent as well as a PKC-independent manner. In turn, activated PKD phosphorylates a wide range of downstream targets at specific sites, subsequently regulating their activity and/or subcellular localization (7). It was well-documented that PKD plays a critical role in the regulation of several important cellular processes, such as DNA synthesis, proliferation, cell survival, adhesion, invasion/migration, motility, and angiogenesis (8). Moreover, PKD signaling has been implicated in several
human tumors, including breast, pancreatic, and prostate cancer, and glioblastoma. For example, PKD2 and PKD3 seem to be highly expressed in breast cancer (9). PKD1 expression level is elevated in human ductal adenocarcinoma of the pancreas compared with normal pancreatic tissues (10). PKD2 has been shown to be an important mediator of induction of various angiogenic factors in human pancreatic cancer cells and in the angiogenic response of the host vasculature (11). Expression of PKD1 and PKD3 has been shown to be elevated in human prostate carcinoma tissue compared with normal prostate epithelial tissue, and advanced stage tumors were found to have increased PKD3 nuclear accumulation (12). PKD2 was also highly expressed in both low-grade and high-grade human gliomas (13).

Given the importance of PKD in tumor biology, investigators have focused on developing novel small molecule inhibitors targeting PKD. Several such agents with anti-cancer activity have been identified, including CID755673, kb-NB142-70, and CRT0066101 (14–16). CID755673 is a non-ATP competitive pan-PKD inhibitor that was discovered in a high throughput screening campaign. This compound blocked phorbol 12-myristate 13-acetate (PMA)-induced activation of PKD1 in human prostate cancer LNCaP cells. Moreover, CID755673 displayed inhibitory effects on cell proliferation, migration, and invasion in prostate cancer cells (16). A series of structural analogs of CID755673 was subsequently developed, including kb-NB142-70, kb-NB165-09, kb-NB165-31, kb-NB165-92, and kb-NB184-02. These agents exhibited at least 2-fold higher potency and improved kinase selectivity when compared with the parent compound (15). The most potent analog, kb-NB142-70, inhibited PKD1, PKD2, and PKD3 enzymatic activity with an IC50 of 28, 59, and 53 nmol/L, respectively. kb-NB142-70 also significantly inhibited cell proliferation, migration, and invasion. Unfortunately, kb-NB142-70 did not exhibit in vivo antitumor activity because of rapid metabolism (17). CRT0066101 is a small molecule PKD-specific inhibitor developed by investigators in the United Kingdom, and it exhibited in vitro antitumor activity in human pancreatic cancer cells. CRT0066101 significantly suppressed neurotensin-induced PKD1/2 activation, blocked NF-kB–mediated cellular proliferation and survival, and induced apoptosis. Moreover, CRT0066101 inhibited Panc-1 cell growth in in vivo xenograft mouse models (14). In addition to CID755673, kb-NB142-70, and CRT0066101, several other pan-PKD inhibitors have been reported in the literature (18, 19).

In this study, we investigated PKD isoform expression in colorectal cancer, evaluated the therapeutic efficacy of targeting PKD in human colorectal cancer, and determined its potential molecular mechanisms of action. We present both in vitro and in vivo evidence showing that CRT0066101 has cytotoxic as well as antitumor activity against human colorectal cancer model systems. These findings provide evidence that PKD may represent a potential target for colorectal cancer chemotherapy.

Materials and Methods

Chemicals and reagents

CRT0066101 was kindly provided by Dr. S. Guha and Cancer Research Technology Inc. For in vitro use, the drug was resuspended in dimethyl sulfoxide (DMSO; Sigma), whereas it was resuspended in 5% sterile dextrose solution for in vivo studies. CID755673 and kb-NB142-70 were synthesized as previously described (15). The DMSO concentration never exceeded 0.1% in any experiment. This dose had no effect on cell growth nor did it affect protein expression. WST-1 was purchased from Roche Diagnostics. PMA and other chemicals were obtained from Sigma.

The following siRNAs were synthesized by Dharmacon Research (ThermoScientific): siPKD2—‘5’-UGAGACACUCUCACUUCC-3’ (ID-004197-05); siPKD3—‘5’-GGAGAGUGUUAACAUUGA-3’ (ID-005029-06); and siCon—‘5’-GGAUACUGCCAAUCUCUGAGG-3’. Tissue culture and human colorectal cancer cell lines

Normal human colon epithelial CAC 841 CoN and FHC cells and the human cancer RKO cell line were obtained from American Type Culture Collection (ATCC). HCT116 p53+/+ and p53−/− cell lines were kindly provided by Dr. B. Vogelstein (20). H630 and H630R1 cells have been maintained in our laboratory after being originally obtained from Dr. A. Gazdar (21). All cell lines, with the exception of FHC, were maintained in RPMI-1640 (Invitrogen) with 10% (v/v) FBS at 37°C in a humidified incubator with 5% CO2. FHC cells were maintained according to ATCC guidelines. HCT116 and RKO cells were authenticated by short tandem repeat profiling at the University of Pittsburgh Cell Culture and Cytogenetics Facility (August 2013). Cells were tested monthly for mycoplasma by the MycoAlert Mycoplasma detection assay (Cambrex BioScience).

Cell viability assay

Human colorectal cancer cells were plated in 96-well plates at a density of 800 to 1,500 cells/well. On the following day, cells were incubated with various concentrations of PKD inhibitors for 72 hours. Cell viability was determined by the WST-1 assay. The IC50 value was defined as the drug concentration that inhibits 50% cell growth compared with the untreated controls and calculated by Graphpad Prism 6.0 software.

Clonogenic assay

HCT116 and RKO cells were seeded in 6-well plates at density of 400 cells/well. On the following day, cells were exposed to various concentrations of CRT0066101 for 24 hours, after which time, the growth medium was then replaced. After 10 to 14 days, cell colonies were fixed with trypan blue solution (75% methanol/25% acetic acid/0.25% trypan blue) for 15 minutes, washed, and air-dried before counting colonies >50 cells.
siRNA transfection

Cells were plated at a density of $2 \times 10^5$ cells/well. On the following day, siRNAs (10 nmol/L) were complexed with Lipofectamine 2000 (Invitrogen) in serum-free RPMI-1640 medium and added to the plated cells. After 48 hours, cells were processed for Western blot analysis or for flow cytometry.

Western blot analysis

Cell lysate protein concentrations were determined using the DC Protein Assay (Bio-Rad). Equal amounts of protein (30 µg) from each cell lysate were resolved on SDS-PAGE using the method of Laemmli and transferred onto 0.45-µm nitrocellulose membranes (Bio-Rad). Membranes were blocked and incubated overnight with primary antibodies at 4°C. The following antibodies were used in the experiments: anti-p-PKD2 ([Ser876] #07–385; Upstate), anti-p-PKD2 (#07–488; Upstate), anti-PKD1 (gift from Dr. P. Storz), anti-PKD3 (#5655; Cell Signaling), anti-p-ERK (#sc-7383; Santa Cruz Biotechnology), anti-ERK (#9272; Cell Signaling), anti-p-AKT (#9542; Cell Signaling), anti-AKT (#9272; Cell Signaling), anti-PARP (#9542; Cell Signaling), anti-α-tubulin (EMD Millipore), and anti-β-tubulin (Sigma). Membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 hour at room temperature. Proteins were detected by the enhanced chemiluminescence method (SuperSignal West Pico substrate; Pierce). Quantitation of signal intensities was performed by densitometry on a Xerox scanner using ImageJ software.

RNA extracts and real-time qRT-PCR analysis

Total RNA was extracted by the guanidine isothiocyanate/phenol/chloroform method (TRizol; Invitrogen). The integrity and purity of the RNA was determined by UV spectrophotometry at OD260/OD280. The first-strand cDNA was synthesized using 1.0 µg total RNA and the iScript Reverse Transcription Supermix for real-time quantitative PCR (qRT-PCR; Bio-Rad). PCR was performed in triplicate using the SsoFast Probes Supermix (Bio-Rad) in a final reaction volume of 10 µL with gene-specific primer/probe sets, and a standard thermal cycling procedure (40 cycles) on a Bio-Rad CFX96 Real-Time PCR System. PKD1, PKD2, PKD3, and 18S RNA levels were assessed using TaqMan Gene Expression real-time PCR assays (Applied Biosystems; assay ID: Hs00177037_m1, ID: Hs00212828_m1, ID: Hs01178657_m1 and Hs03928990_g1, respectively). A cDNA array of 24 human colon tumors (Colon Cancer cDNA Array III; Origene) was also analyzed for PKD isoform expression (normalized by β-actin RNA levels; assay ID: Hs01060665_g1). Results were expressed as the threshold cycle (Ct). The relative quantification of the target transcripts was determined by the comparative Ct method (ΔΔCt) according to the manufacturer’s protocol. The 2−ΔΔCt method was used to analyze the relative changes in gene expression. Control experiments were conducted without reverse transcription to confirm that the total RNA was not contaminated with genomic DNA.

Flow cytometry

HCT116 and RKO cells were seeded in 6-well plates at a density of $4 \times 10^5$ cells/well. After exposure to CRT0066101 for 24 hours, cells were harvested, washed twice with PBS, resuspended with 1× binding buffer, stained with FITC Annexin V Apoptosis Detection Kit (BD Biosciences), and analyzed on the BD Accuri C6 Flow Cytometer (BD Accuri Cytometers Inc.) at the UPCI Cytometry Facility. For cell cycle analysis, cells were washed with PBS and fixed with 70% ethanol overnight. Cells were washed with PBS, and then treated with 1 mg/mL RNase A for 30 minutes at 37°C. Cells were incubated with propidium iodide (1 mg/mL) for 45 minutes before detection by flow cytometry.

NF-κB activity assay

HCT116 and RKO cells were seeded in 24-well plates at a density of $1 \times 10^5$ cells/well. On the following day, pGL3-Luc-NF-κB or pGL3-Luc DNA (0.5 µg) was cotransfected with 0.1 µg of Renilla luciferase plasmid DNA into cells using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). The Renilla luciferase plasmid DNA was used as an internal control for transfection efficiency. After 6 hours, transfection medium was changed, and on the following day, cells were incubated for 2 hours with various concentrations of PKD inhibitors followed by addition of 50 ng/mL TNF-α for an additional 5 hours. Firefly luciferase values were normalized with renilla activity and the reporter assays were performed in triplicate. To generate RKO cells that stably express NF-κB-driven luciferase, RKO cells were transfected with pGL3-Luc-NF-κB. After 48 hours, 400 µg/mL hygromycin B was added to the cells. After 2 weeks of antibiotic selection, the heterogeneous RKO cells were incubated for 2 hours with various concentrations of PKD inhibitors followed by the presence or absence of 100 nmol/L PMA for an additional 5 hours. Firefly luciferase activity was then determined as described above and was normalized to total soluble protein.

Xenograft mouse model experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. HCT116 cells (<70% confluent) were harvested, and $5 \times 10^5$ cells in 0.1 mL of medium were implanted subcutaneously on the back of athymic nude female mice. When the tumor size reached approximately 100 mm³, mice were randomized into the following groups (5 mice per group): (A) control (vehicle; 5%...
dextrose); (B) 40 mg/kg, (C) 80 mg/kg, and (D) 120 mg/kg CRT0066101 (dissolved in 5% dextrose) administered orally once daily. Therapy was administered for 3 weeks, and animals were sacrificed on day 21 after treatment with CRT0066101. Tumor volume was measured as \( V = \frac{1}{2}ab^2 \), in which “a” and “b” represents length and width of tumor (22). Tumor volumes were monitored 3 times per week. At the time the animals were euthanized, half of the tumor tissue was fixed with formalin and paraffin-embedded for immunohistochemistry. The other half was snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). Tissue slides were processed by the Department of Pathology Development Laboratory and the Tissue and Research Pathology Services at the University of Pittsburgh for Ki67 (#9027; Cell Signaling), in situ terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (APOPTAG Peroxidase Kit; Chemicon), p-ERK (#4370; Cell Signaling), and M30 (#12140322001; Roche).

**Statistical analysis**

Data were expressed as the mean ± SD. Statistical analysis of the data was performed using one-way ANOVA (SPSS software). \( P < 0.05 \) was considered statistically significant.

**Results**

### PKD expression in colorectal cancer

We analyzed the expression of the 3 different PKD isoforms in 2 normal epithelial colon cell lines (FHC and 841) and 3 human colorectal cancer cell lines. As shown in Fig. 1A and B, PKD1 was only expressed in normal colon cells and not in colorectal cancer cells. In contrast, PKD2 and PKD3 were expressed, at the protein and mRNA level, in all cell lines. To provide further support for the differential level of expression of the respective PKD RNA isoforms in human colorectal cancer, we used the CellMiner web-based program to interrogate RNA transcript patterns in the NCI-60 cell panel (23). The 7 human colorectal cancer cell lines in the panel are COLO205, HCC2998, HCT116, HCT15, HT29, KM12, and SW620. The level of PKD1 RNA expression was significantly reduced below the median transcript expression (Z-score = 0) in all cell lines, whereas PKD3 RNA expression was significantly increased above the median transcript expression (Z-score = 3).
expression was somewhat more variable (Supplementary Fig. S1). In sharp contrast, expression of PKD2 RNA was elevated in 5 of 7 cell lines. A search of the Broad-Novartis Cancer Cell Line Encyclopedia database (24), which contains genetic information on 1,000 cell lines, revealed that human colorectal cancer cell lines express the lowest PKD1 mRNA levels among all the cell lines (Supplementary Fig. S2). PKD2 expression in the 61 colorectal cancer cell lines was slightly higher than the mean mRNA levels (8.36 vs. 8.26; RMA, log2) whereas PKD3 expression was somewhat lower than the mean (8.07 vs. 8.42; RMA, log2).

To provide further support for the potential clinical relevance of PKD expression, we performed qPCR analysis on cDNAs obtained from 24 human colon tumors (Origene cDNA array). After estimation of all PKD isoforms, we observed a nearly identical RNA expression pattern with PKD2 being the dominantly expressed isoform (Fig. 1C). Taken together, these data suggest that PKD2 is the most abundant isoform in colorectal cancer.

We next investigated the effect of pan-PKD inhibitors on PKD activation in the human colorectal cell lines. Given the high level of PKD2 expression in HCT116 and RKO and the availability of a specific p-PKD2 antibody, we monitored phosphorylation of the PKD2 isoform by Western blot analysis. Cells were incubated with PKD inhibitors followed by stimulation with PMA, a known activator of the PKC/PKD pathway. As shown in Fig. 1D and E, treatment with CRT0066101 significantly inhibited PKD2 phosphorylation in a dose-dependent manner. The highest concentration of CRT0066101 (10 μmol/L) almost completely blocked PKD2 activation in HCT116 and RKO cells. The concentration that inhibited 50% of PKD2 activation was 2 and 3 μmol/L, respectively. Treatment of RKO cells with a different PKD inhibitor, kb-NB142-70, also decreased p-PKD2 levels, but to a much lesser extent than CRT0066101 (Supplementary Fig. S3A; IC50, 38 μmol/L).

**Effect of PKD inhibition on growth of human colorectal cancer cells**

To determine the potential impact of PKD suppression on cell growth, a panel of colorectal cancer cell lines was treated with different PKD inhibitors. Cells were exposed to CRT0066101, CID755673, and kb-NB142-70 for 72 hours, and cell proliferation was determined by the WST-1 assay. CRT0066101 exhibited low μmol/L IC50 values against all colorectal cancer cell lines in the panel (Table 1). kb-NB142-70 had similar inhibitory effects on cell growth albeit with slightly higher IC50 values. In contrast, CID755673 was significantly less potent, as had been shown previously observed with different human cancer model systems (16). Growth inhibition was similar in both p53+/− and p53−/− HCT116 cells suggesting that the cytotoxic effects of these molecules are mediated through p53-independent pathways. These compounds were also able to maintain their inhibitory activity in cells that are resistant to both chemotherapy and radiation therapy. Of note, the 5-FU-resistant H630R1 cells were as sensitive to the PKD inhibitors as parental H630 cells. In addition to the WST-1 assay, we utilized the clonogenic assay to determine the effect of CRT0066101 on HCT116 and RKO clonogenic growth. CRT0066101 effectively decreased colony number in a dose-dependent manner (Supplementary Fig. S4).

**Effect of PKD inhibition on cell-cycle distribution and apoptosis**

Given the growth inhibitory activity of CRT0066101 on human colorectal cells, we evaluated the potential mechanisms of action of PKD inhibition in these cells. To determine the effect of CRT0066101 on distribution of the cell cycle, HCT116 and RKO cells were treated with CRT0066101 for 24 hours, stained with propidium iodide, and analyzed by flow cytometry. As shown in Fig. 2A, CRT0066101 blocked cell-cycle progression at the G2–M phase in a dose-dependent manner. Similar results were obtained with kb-NB142-70 (Supplementary Fig. S3B and S3C). This block at the G2–M checkpoint coincided with a decrease in the fraction of cells in the G1 population. We also observed a significant increase in the sub-G1 phase in both cell lines suggesting that CRT0066101 may induce apoptosis of human colorectal cancer cells. To further investigate the potential impact of this agent on inducing apoptosis, cells were treated with CRT0066101 for 24 hours, stained with FITC-Annexin V and propidium iodide and analyzed by flow cytometry. As shown

### Table 1. Effect of PKD inhibitors on cell proliferation

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>PKD inhibitor IC50 (μmol/L)</th>
<th>CRT0066101</th>
<th>kb-NB142-70</th>
<th>CID755673</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKO</td>
<td></td>
<td>0.90 ± 0.17</td>
<td>2.82 ± 0.67</td>
<td>24.68 ± 5.00</td>
</tr>
<tr>
<td>HCT116 (p53+/−)</td>
<td></td>
<td>0.77 ± 0.25</td>
<td>2.69 ± 0.07</td>
<td>15.51 ± 2.55</td>
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<tr>
<td>HCT116 (p53−/−)</td>
<td></td>
<td>1.01 ± 0.06</td>
<td>3.08 ± 0.38</td>
<td>17.52 ± 1.80</td>
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<tr>
<td>H630</td>
<td></td>
<td>1.28 ± 0.18</td>
<td>4.33 ± 2.18</td>
<td>46.7 ± 27.65</td>
</tr>
<tr>
<td>H630R1</td>
<td></td>
<td>1.56 ± 0.34</td>
<td>8.35 ± 4.31</td>
<td>35.28 ± 5.42</td>
</tr>
</tbody>
</table>

NOTE: IC50 values represent the concentration of drug that suppressed cell growth by 50%. Values represent the mean ± SD from 3 to 5 determinations.
Figure 2. Effect of PKD inhibition on cell-cycle distribution and apoptosis. HCT116 and RKO cells were incubated with various concentrations of CRT0066101 (0.3–10 μmol/L) for 24 hours. A, cell-cycle distribution was detected by flow cytometry. Cycle percentages represent the mean ± SD from 3 individual experiments using HCT116 and RKO cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, apoptotic cells were detected by flow cytometry. Apoptotic cell percentages represent the mean ± SD from 3 individual experiments. ###, P < 0.001 early apoptosis versus control; ####, P < 0.001 late apoptosis versus control. C, expression of cleaved-PARP and cleaved caspase-3 was determined by Western blot analysis after 24 hours exposure to CRT0066101. D, cells were transfected with 10 nmol/L siRNAs for 48 hours, and expression of cleaved PARP and caspase-3 was analyzed by Western blot analysis. E, cells were transfected with 10 nmol/L siRNAs for 48 hours and processed for apoptotic cell detection by flow cytometry.
in Fig. 2B, CRT0066101 significantly induced apoptosis in both cell lines in a dose-dependent manner. A different molecule kb-NB142-70 induced slightly lower levels of apoptosis (Supplementary Fig. S3D and S3E). We then investigated the effect of drug treatment on the expression of other markers of apoptosis. As determined by Western blot analysis, CRT0066101 treatment (1–3 μmol/L) resulted in cleavage of both PARP and caspase-3, respectively (Fig. 2C). Because the PKD inhibitors suppress all PKD isoforms, we transfected isoform-specific siRNAs into colorectal cancer cells to identify which isoform might be responsible for the growth inhibitory activity of the small molecule inhibitors. Each siRNA specifically and potently suppressed only the intended targeted isoform (Fig. 2D). Knockdown of PKD2, but not PKD3, resulted in cleavage of PARP and caspase-3. Furthermore, transfection of PKD2 siRNA induced apoptosis as determined by flow cytometry, whereas PKD3 siRNA transfection had no effect on Annexin V/propidium iodide staining (Fig. 2E).

**Effect of CRT0066101 on key cellular survival pathways**

The PI3K-AKT, MAPK, and NF-κB signaling pathways are well-established survival pathways in human colorectal cancer. Constitutive activation of each of these pathways, as a result of point mutations, has been shown to increase cancer cell proliferation and drug resistance (25). It has also been demonstrated that each of these pathways are downstream of PKD signaling (26). We next determined whether any of these pathways might represent active downstream components of PKD signaling in colorectal cancer cells. We treated human HCT116 and RKO cells with CRT0066101 for 24 hours, and then determined expression of p-AKT, p-ERK, total AKT, and total ERK by Western blot analysis. CRT0066101 treatment resulted in a significant reduction in expression of p-AKT and p-ERK, with no effect on total AKT and ERK protein levels (Fig. 3A–C). Transfection of PKD2 siRNA resulted in a similar decrease in p-AKT expression in RKO cells and a decrease in p-ERK expression in both cell lines (Fig. 3D). In contrast, treatment with the PKD3 siRNA did not alter these pathways. We next detected the effect of PKD inhibition on NF-κB activity. HCT116 and RKO cells were transiently transfected with a luciferase plasmid under the control of the NF-κB response element. After incubation of PKD inhibitors for 2 hours, cells were stimulated by TNF-α (50 ng/mL) for an additional 5 hours. NF-κB activity was determined by the dual luciferase assay. As shown in Fig. 3E, CRT0066101 effectively suppressed, in a dose-dependent manner, the activation of NF-κB. Significant inhibition was observed after treatment with 1 μmol/L CRT0066101. In contrast, higher doses of kb-NB142-70 were required to obtain similar suppression of NF-κB activity (Supplementary Fig. S3F).

To determine whether PKD suppression can alter basal expression of NF-κB, RKO cells were stably transfected with the NF-κB–driven luciferase plasmid. Addition of CRT0066101 to these cells in the absence of an inducer resulted in a dose-dependent inhibition of basal NF-κB activity (Fig. 3F). Although TNF-α activates the NF-κB pathway, it does not directly induce the PKC/PKD pathway (data not shown). Thus, cells preincubated with CRT0066101 were stimulated with PMA, a known PKD stimulator. As shown in Fig. 3F, PMA-induced NF-κB activation was suppressed with the PKD inhibitor. Taken together, our findings suggest that PKD inhibition and in particular PKD2, results in suppression of key signaling pathways in human colorectal cancer, including AKT, ERK, and NF-κB.

**Antitumor activity of PKD small molecule inhibitors**

Our *in vitro* results suggest that PKD plays an active role in colorectal cancer growth and proliferation. As CRT0066101 is an orally bioavailable PKD inhibitor, we further evaluated the *in vivo* antitumor activity of CRT0066101 using HCT116 tumor-bearing athymic nude mice. Mice were administered different oral doses of CRT0066101 (40, 80, and 120 mg/kg) once daily for 3 weeks. At the end of the 21-day treatment period, tumor volume was decreased 55.6%, 65.2%, and 69.5%, respectively, as compared with control, vehicle-treated mice (Fig. 4A and C). Although the reduction in mean tumor volume seemed to be dose dependent, this effect was not statistically significant. As compared with the vehicle-treated group, significant antitumor activity for each dose was achieved on day 16, day 12, and day 9, respectively. No gross toxicities were evident as determined by body weight, even at the highest dose (Fig. 4B). We also determined the potential effect of drug treatment on PKD2 activation within the tumor xenografts. As seen in Fig. 4D, treatment with CRT0066101 resulted in a dose-dependent suppression of p-PKD2 in the xenograft tumors. Tumor xenografts were examined for additional markers of growth and apoptosis. For these studies, Ki67 expression and the level of TUNEL and M30 staining was investigated. Ki67 is a well-established marker for cell proliferation. Treatment with the lowest CRT0066101 dose used in this experiment, 40 mg/kg, resulted in a significant reduction in Ki67 expression (Fig. 5). In addition, expression levels of p-ERK were investigated. We observed a dose-dependent inhibition of p-ERK expression, which correlated with our *in vitro* results. The TUNEL assay detects DNA fragmentation as a result of apoptotic signaling cascades. CRT0066101 treatment significantly increased TUNEL staining in a dose-dependent manner (Fig. 5). To verify that the increased TUNEL staining signified apoptosis and not necrosis, staining for M30, a caspase-cleaved cytokeratin 18, was performed. As shown in Fig. 5, CRT0066101 administration significantly increased M30 staining suggesting that the PKD inhibitor induced apoptosis in our *in vivo* colorectal cancer model.

**Discussion**

It is now well-established that PKD signaling mediates several key cellular processes, including DNA synthesis,
Figure 3. Effect of CRT0066101 on downstream cell survival pathways. A, cells were exposed to CRT0066101 for 24 hours, and protein expression was determined by Western blot analysis. B, semiquantitative analysis of Western blots for p-ERK/total ERK. C, semiquantitative analysis of Western blots for p-AKT/total AKT. Values represent the mean ± SD from at least 3 individual blots. E, HCT116 and RKO cells were transiently transfected with a luciferase plasmid under the control of a NF-κB response element. On the following day, cells were treated with CRT0066101 for 2 hours, and then stimulated with TNF-α for an additional 5 hours. NF-κB activity was determined by the dual-luciferase assay. F, RKO cells stably expressing NF-κB luciferase were treated with CRT0066101 for 2 hours, and then stimulated with PMA (100 nmol/L) for an additional 5 hours. Luciferase values represent the mean ± SD from 3 to 5 separate experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus untreated cells (B, C), TNF-α-stimulated cells (E), unstimulated cells (-PMA; F), or PMA-stimulated cells (F). ‡, P < 0.001 versus untreated cells (E).
proliferation, survival, adhesion, invasion/migration, motility, and angiogenesis (8). This pathway has also been implicated in a broad range of human tumors. Moreover, there is evidence suggesting that different PKD isoforms may be associated with specific cancers (27). In the literature, relatively little is known about the precise role of PKD in colorectal cancer. For this reason, we initially determined the respective mRNA and protein expression levels of each PKD isoform in normal human colon and colorectal cancer cell lines. PKD1 expression was detectable only in the normal colon cell lines. However, significant expression of PKD2 and PKD3 was observed in all
cell lines. These findings were validated in a search of the CellMiner and CCLE databases and subsequently confirmed with expression analysis in human colon tumor samples. The absence of PKD1 in colorectal cancer was somewhat puzzling as it had been previously reported that PKD1 is abundantly expressed in mouse intestinal cells and that expression of this particular isoform is necessary for DNA synthesis and cellular migration (28). It is conceivable that the loss of PKD1 expression may be the result of epigenetic silencing as normal intestinal cells transform into cancer cells. In support of this possibility, investigators have also identified loss of PKD1 expression in gastric and breast cancer resulting from epigenetic regulatory mechanisms (9, 29). With this in mind, we attempted to reexpress PKD1 with the use of DNA methyltransferase inhibitors (decitabine and RG108) but were unsuccessful in doing so (data not shown). The CCLE database states that HCT116 cells have a point mutation in PKD1 (S625N) and RKO cells have 2 point mutations (G779D, C853R). However, at this time, it is unclear as to the potential impact of these discrete point mutations on protein expression and/or stability.

The development and evaluation of small molecule inhibitors directed against novel targets, such as PKD, would seem to be critically important for identifying new therapeutic options that can ultimately improve patient response and outcome. To date, several PKD inhibitors have been developed with activity against a wide variety of tumor types (8). Our studies reveal that 2 of these inhibitors, kb-NB142-70 and CRT0066101, exhibited significant activity against a wide range of human colorectal cancer cell lines. Interestingly, these PKD inhibitors equally suppressed growth of multidrug-resistant cells as well as p53-deficient cells, suggesting that PKD inhibitors may be able to overcome chemoresistance and radioresistance mechanisms.

With respect to the potential mode of action, our studies show that PKD inhibition resulted in cell-cycle arrest at the G2–M phase, a finding that was previously demonstrated in human prostate cancer cells (15). Similarly, Kienzle and colleagues demonstrated that PKD1 and PKD2 are necessary in the G2 phase of the cell cycle in HeLa cells (30). Interestingly, siRNA knockdown of PKD3 resulted in accumulation of prostate cancer cells in G2–G1 phase (12). Studies by Azoitei and colleagues showed that glioblastoma cells accumulate in G2–G1 phase after PKD2 suppression (13). Thus, in addition to cell-specific expression, the different PKD isoforms may play different roles in mediating cell division and proliferation.

Using our human colorectal cancer model systems, we observed that PKD inhibition, through the use of either small molecule inhibitors or siRNAs, resulted in significant induction of apoptosis. This effect was detected by both Annexin V/propidium iodide staining and Western blot analysis of cleaved PARP and caspase-3. In addition to induction of apoptotic pathways, PKD inhibition suppressed Akt and ERK signaling. Previously, PKD has been shown to mediate the MEK/ERK/RSK pathway and promote cell proliferation through a stimulatory effect on GPCR (31). The pro-proliferative effects of PKD in cancer cells are associated with activation of both ERK1/2 and AKT. In human prostate cancer, PKD3 modulated both the extent and duration of ERK1/2 activation (12). In this study, we showed that the small molecule inhibitor CRT0066101 strongly suppressed activation of ERK in human colorectal cancer. This agent also significantly blocked AKT activation. Transfection of PKD2 siRNA resulted in similar effects on these 2 downstream pathways. Other key regulators of cell survival, proliferation, and motility are the NF-kB transcription factors. Previous studies have shown that PKD is a mediator of NF-kB induction in a variety of cells exposed to GPCR agonists or oxidative stress (32). PKD2 gene silencing dramatically blocked LPA-stimulated NF-kB promoter activity in non-transformed human colonic epithelial NCM460 cells (33). However, they observed no decrease in ERK signaling. PKD2 has also been implicated in mediating NF-kB activation by Bcr-Abi in myeloid leukemia cells (34). In keratinocytes, in which phorbol esters are major tumor promoters, PKDs stimulate proliferation and prevent differentiation. These findings suggest that PKDs and, in particular, PKD2 may play an important role in phorbol ester-sensitive tumors, such as skin tumors and colon cancer. PMA induces NF-kB activation, and PKDs has been shown to promote cell survival through activating NF-kB signaling pathway in response to oxidative stress (8). Thus, it is conceivable that the prosurvival effects of PKD2 in colorectal cancer cells, in response to PMA treatment, may be partly attributable to NF-kB activation. Herein, we confirmed that suppression of PKD with small molecule inhibitors leads to significant inhibition of NF-kB activity in colorectal cancer. Taken together, these 3 key survival pathways, Akt, ERK, and NF-kB, each of which are located downstream of PKD signaling, seem to be critical for the cytotoxic activity of PKD inhibitors in human colorectal cancer.

To date, several PKD inhibitors have been studied in in vivo animal models. As one example, kb-NB142-70, demonstrated significantly improved in vitro activity compared with the structurally related CID755673, but exhibited no antitumor activity in vivo, which is most likely because of rapid metabolism (17). Alternatively, a naphthyridine-based PKD inhibitor showed PKD inhibition and suppression of PKD-dependent downstream pathways in an in vivo rat model (35). The PKD inhibitors kb-NB142-70 and CRT0066101 displayed similar activities in our in vitro models, and CRT0066101 also demonstrated significant antitumor activity in 2 pancreatic animal models (14). Our data confirm the antitumor activity of CRT0066101 and further demonstrate that colon cancer xenografts are responsive to in vivo inhibition of the PKD pathway. With the colorectal cancer models that were used in this study, we observed greater tumor suppression at lower doses than previously reported, suggesting that colon cancer may be more susceptible to blockage of this growth-mediating pathway. However, given the
critical roles of PKDs in both endothelial and epithelial cells, a major concern is that the use of pan-PKD inhibitors may lead to off-target effects. Several PKD inhibitors have demonstrated such off-target effects against other kinases (19, 35). It is conceivable that the orally bioavailable inhibitor CRT0066101 may bind to and inhibit other kinases. Notably, the 2-(4-aminopyrimidin-2-yl)phenol moiety in CRT0066101 is also present in small molecule kinase inhibitors targeting the proto-oncogene serine/threonine-protein kinase (Pim-1), checkpoint kinase 2, inhibitor of NF-κB kinase subunit β, tropomyosin-related kinases and others (36–39). However, specific PKD isoform targeting with either siRNAs or antisense molecules can suppress in vivo tumor growth (11, 40). Thus, the development of small molecule inhibitors and/or nucleic acid–based molecules, such as siRNAs, targeting specific PKD isoforms is warranted.

In conclusion, we report that small molecule inhibitors directed against PKD signaling exhibited potent in vitro cytotoxic and in vivo antitumor activity. The bio-logic effects of these agents seem to be mediated by inhibition of PKD2 activation, G2-M phase arrest, induction of apoptosis, and inhibition of AKT, ERK, and NF-κB signaling pathways. These studies are important as they provide support for the potential role of PKD as a novel target for cancer chemotherapy. Moreover, they provide a rational basis for the design and development of novel agents that may act either alone or in combination with presently available anti-cancer agents to enhance clinical activity and/or overcome cellular drug resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Wei, E. Chu, P. Wipf, J.C. Schmitz Development of methodology: N. Wei, E. Chu, J.C. Schmitz Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Wei, J.C. Schmitz Writing, review, and/or revision of the manuscript: N. Wei, E. Chu, P. Wipf, J.C. Schmitz Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.C. Schmitz

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References


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