

Research Article

A Novel Small-Molecule Inhibitor of Protein Kinase D Blocks Pancreatic Cancer Growth *In vitro* and *In vivo*

Kuzhuvelil B. Harikumar¹, Ajaikumar B. Kunnumakkara¹, Nobuo Ochi², Zhimin Tong², Amit Deorukhkar³, Bokyoung Sung¹, Lloyd Kelland^{7†}, Stephen Jamieson⁷, Rachel Sutherland⁷, Tony Raynham⁷, Mark Charles⁷, Azadeh Bagherzadeh⁷, Caroline Foxton⁷, Alexandra Boakes⁷, Muddasar Farooq⁷, Dipen Maru⁴, Parmeswaran Diagaradjane³, Yoichi Matsuo², James Sinnott-Smith⁶, Juri Gelovani⁵, Sunil Krishnan³, Bharat B. Aggarwal¹, Enrique Rozengurt⁶, Christopher R. Ireson⁷, and Sushovan Guha²

Abstract

Protein kinase D (PKD) family members are increasingly implicated in multiple normal and abnormal biological functions, including signaling pathways that promote mitogenesis in pancreatic cancer. However, nothing is known about the effects of targeting PKD in pancreatic cancer. Our PKD inhibitor discovery program identified CRT0066101 as a specific inhibitor of all PKD isoforms. The aim of our study was to determine the effects of CRT0066101 in pancreatic cancer. Initially, we showed that autophosphorylated PKD1 and PKD2 (activated PKD1/2) are significantly upregulated in pancreatic cancer and that PKD1/2 are expressed in multiple pancreatic cancer cell lines. Using Panc-1 as a model system, we showed that CRT0066101 reduced bromodeoxyuridine incorporation; increased apoptosis; blocked neurotensin-induced PKD1/2 activation; reduced neurotensin-induced, PKD-mediated Hsp27 phosphorylation; attenuated PKD1-mediated NF- κ B activation; and abrogated the expression of NF- κ B-dependent proliferative and prosurvival proteins. We showed that CRT0066101 given orally (80 mg/kg/d) for 24 days significantly abrogated pancreatic cancer growth in Panc-1 subcutaneous xenograft model. Activated PKD1/2 expression in the treated tumor explants was significantly inhibited with peak tumor concentration (12 μ mol/L) of CRT0066101 achieved within 2 hours after oral administration. Further, we showed that CRT0066101 given orally (80 mg/kg/d) for 21 days in Panc-1 orthotopic model potently blocked tumor growth *in vivo*. CRT0066101 significantly reduced Ki-67-positive proliferation index ($P < 0.01$), increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive apoptotic cells ($P < 0.05$), and abrogated the expression of NF- κ B-dependent proteins including cyclin D1, survivin, and cIAP-1. Our results show for the first time that a PKD-specific small-molecule inhibitor CRT0066101 blocks pancreatic cancer growth *in vivo* and show that PKD is a novel therapeutic target in pancreatic cancer. *Mol Cancer Ther*; 9(5); 1136–46. ©2010 AACR.

Introduction

Protein kinase D (PKD) is a new family of serine/threonine kinases composed of PKD1, PKD2, and PKD3 and is characterized by distinct structural features and enzymological properties (reviewed in ref. 1). PKD family members are known effectors of diacylglycerol-regulated signal transduction pathways and are activated by activation loop phosphorylation through protein kinase C (PKC)-dependent (1, 2) and PKC-independent (3) path-

ways. We and others showed that activated PKD1 and PKD2 autophosphorylate at the COOH-terminal end, corresponding to Ser-910 in human PKD1 (Ser-916 in murine PKD1) and Ser-876 in human PKD2 (1). Accumulating evidence suggests that PKD family members play a critical role in the regulation of several cellular processes and activities, including chromatin organization, Golgi function, gene expression, cell survival, adhesion, motility, differentiation, DNA synthesis, and proliferation (reviewed in ref. 1). PKD1 activation also initiates the

Authors' Affiliations: Departments of ¹Experimental Therapeutics, ²Gastroenterology, Hepatology, and Nutrition, ³Radiation Oncology, ⁴Pathology, and ⁵Experimental Diagnostic Imaging, The UT MD Anderson Cancer Center, Houston, Texas; ⁶Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine, Los Angeles, California; and ⁷Cancer Research Technology Discovery Laboratories, Wolfson Institute for Biomedical Research, London, United Kingdom

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[†] Deceased.

Corresponding Authors: Sushovan Guha, Department of Gastroenterology, Hepatology, and Nutrition, Unit 436, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-745-7566; Fax: 713-563-4398. E-mail: sguha@mdanderson.org or Christopher R. Ireson, Cancer Research Technology Discovery Laboratories, Wolfson Institute for Biomedical Research, Gower Street, London WC1E 6BT. Phone: 44-020-7679-0960; Fax: 44-020-7679-6718. E-mail: cireson@cancertechnology.com

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NF- κ B signaling pathway, triggering cell survival responses (4). Overexpression of PKD1 or PKD2 enhanced cell cycle progression and DNA synthesis in Swiss 3T3 fibroblasts (5). PKD is implicated in multiple pathologic conditions including regulation of cardiac gene expression and contractility (6). Consequently, the development of specific PKD family inhibitors would be useful for defining the physiologic roles of PKD as well as for developing novel therapeutic approaches in a variety of pathologic conditions.

Neuropeptides, including neurotensin, and growth factors promote activation of PKD family members in multiple neoplasias including pancreatic cancer, a devastating disease with an overall 5-year survival rate of only 3% to 5% (7, 8). We showed that G protein-coupled receptor (GPCR) agonists including neurotensin-stimulated, PKD-dependent mitogenic signaling pathways in pancreatic cancer (9) and more recently that PKD1 overexpression facilitated DNA synthesis and proliferation in pancreatic cancer cells (10). PKD1 significantly induced resistance to CD95-dependent apoptosis (11) and phosphorylated Hsp27 in pancreatic cancer (12), which is implicated in drug resistance in these cells (13). PKD also plays a potential role in cancer cell invasion and motility (14) and is necessary for tumor-associated angiogenesis (2). As PKD plays a crucial role in tumorigenesis including pancreatic cancer, we initiated a PKD inhibitor discovery program to further unravel its biological functions.

Here, we describe antitumor activities of a small-molecule PKD family-specific inhibitor CRT0066101 in pancreatic cancer. We showed that activated PKD1/2 (i.e., autophosphorylated at the COOH-terminal end) are overexpressed in pancreatic cancer compared with normal pancreatic ducts and that these PKD family members are also abundantly expressed in multiple pancreatic cancer cell lines compared with immortalized human pancreatic duct epithelial (HPDE) cell line. Using Panc-1 cells as our model system, we showed that CRT0066101 significantly blocked proliferation, induced apoptosis, reduced neurotensin-induced PKD1/2 activation, abrogated activation of PKD1/2-induced NF- κ B, and blocked NF- κ B-dependent gene products essential for cell proliferation and survival. Further, CRT0066101 blocked Panc-1 cell proliferation and growth in multiple xenograft models. CRT0066101 reduced proliferation index (Ki-67-positive cells), increased apoptosis [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells], and abrogated the expression of several NF- κ B-dependent prosurvival proteins in tumor explants. Our results showed that CRT0066101 is a novel PKD-specific inhibitor that blocks pancreatic cancer growth both *in vitro* and *in vivo*.

Materials and Methods

Please see Supplementary Materials and Methods for further details.

Cell cultures and reagents. Pancreatic cancer cell lines including Panc-1 were obtained either from the American Type Culture Collection or from Cancer Research UK (CR-UK). They were cultured either in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin or in DMEM from CR-UK supplemented with 10% FCS (PAA). The HPDE cells were generous gifts from Dr. Ming-Sound Tsao (University of Toronto, Ontario, Canada; refs. 15, 16). These cells were cultured in a keratinocyte serum-free medium supplied with 5 ng/mL epidermal growth factor and 50 μ g/mL bovine pituitary extract (Invitrogen). Cells were regularly tested for Mycoplasma and were found to be negative. Antibodies to Hsp27, pS82-Hsp27, pS152/156-MARCKS, and pS916-PKD1/2 antibodies were purchased from Cell Signaling Technology. Survivin and β -actin antibodies were obtained from R&D Systems and Sigma, respectively. Antibodies to PKD-1/2 (total), cyclin D1, cIAP1, Bcl-xL, and Bcl-2 were purchased from Santa Cruz Biotechnology.

Immunohistochemistry. Formalin-fixed, paraffin-embedded pancreatic cancer tissue microarrays (US Biomax) were stained with monoclonal pS916-PKD1/2 antibody (Epitomics) at a 1:10 dilution overnight at 4°C as previously described (17). This monoclonal antibody specifically recognizes activated PKD1/2.

Biochemical IC₅₀ determination and specificity. Specificity of CRT0066101 was performed by *in vitro* kinase assays using a commercial kinase profiling service (Millipore UK Ltd). The IC₅₀ of CRT0066101 on PKD1-3 was determined *in vitro* using immobilized metal ion affinity-based fluorescence polarization (MDS Analytical Technologies Ltd).

Fast activated cell-based ELISA assay. The fast activated cell-based ELISA assay (Active motif) was used to measure effects of CRT0066101 on pS916-PKD expression *in vitro*.

Bromodeoxyuridine incorporation assay. Cell proliferation was measured using bromodeoxyuridine (BrdUrd) proliferation kits (Thermo Scientific Cellomics Europe) in accordance with the manufacturer's instructions.

Apoptosis assay. The Meso Scale Discovery and the Multiplex Apoptosis Panel kit were used to determine levels of cleaved caspase-3 (MSD).

Cell viability assay. Cell viability was measured using the CellTiter Aqueous One Solution Cell Proliferation Assay kit (Promega) as previously described (17).

Electrophoretic mobility shift assay. To determine activation of NF- κ B, electrophoretic mobility shift assay (EMSA) was done using nuclear extracts of Panc-1 cells either transfected with control (Panc-1) or PKD-1 overexpressing (Panc-1-PKD1) vectors before treatment with DMSO or CRT0066101 as previously described (18).

NF- κ B luciferase reporter assay. Panc-1 cells (1×10^5) were plated in 24-well plates 16 hours before transfection. A total of 0.76 μ g of pGL3-Luc-NF- κ B or pGL3-Luc DNA was then cotransfected with 0.4 μ g of *Renilla* luciferase vector DNA (internal control, Promega) into

Panc-1 cells using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Luminescence values were normalized with *Renilla* activity and the reporter assays were done in triplicate.

Animals. Male athymic *nu/nu* mice (4-wk-old) and CD-1 mice were either obtained from the breeding colony of the Department of Experimental Radiation Oncology at the University of Texas MD Anderson Cancer Center (UTMDACC) or from Cancer Research Technology, UK (CR-UK). Before initiating experiments, we acclimatized all mice to a pulverized diet for 3 days. All the mice were weighed daily during the course of experiments. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at UTM-DACC or at CR-UK.

Maximum tolerated dose assay. Before examining the efficacy of the PKD inhibitor CRT0066101, the maximum tolerated dose of the drug was established in CR-UK *nu/nu* mice and determined to be 80 mg/kg/d.

Heterotopic (subcutaneous) pancreatic cancer model. CR-UK *nu/nu* mice were inoculated s.c. into the left and right flanks with 5×10^6 cells of Panc-1 cells in 100 μ L PBS. Tumors were allowed to grow to their optimal size as measured by calipers.

Determination of mouse pharmacokinetics and efficacy of CRT0066101 in a heterotopic pancreatic cancer model. CD-1 mice were given a daily oral dose of 80 mg/kg for 5 days and blood was removed by cardiac puncture under terminal anesthesia for the measurement of plasma concentration. For the heterotopic xenograft model, CR-UK *nu/nu* mice were s.c. injected with 5×10^6 Panc-1 cells. Nineteen days after implantation of Panc-1 cells, tumor areas were on average 0.3 cm² and were randomized into the following groups ($n = 8$ mice per group): (a) vehicle (control) 5% dextrose administered by oral gavage once daily and (b) 80 mg/kg CRT0066101 dissolved in 5% dextrose administered by oral gavage once daily. Tumors were measured in two dimensions every 2 to 3 days by calipers and area was calculated by multiplying length by width. Therapy was given until tumors reached their designated size limits (1.44 cm²) or until day 24 in the CRT0066101-treated group. Final tumor areas were compared among groups using a Student's *t* test and Fisher's exact test with $P < 0.05$.

Measurement of active PKD (pS916-PKD) in heterotopic tumor explants. CR-UK *nu/nu* mice were inoculated with 5×10^6 cells per flank and left to grow until the tumors reached ~ 0.3 cm² in size. Mice were treated as indicated for 5 days and sacrificed at 0, 2, 6, and 24 hours after the last treatment. Tumors were excised and lysates were prepared for analysis by Western blot using the pS916-PKD1/2 antibody (Cell Signaling Technology).

Measurement of CRT0066101 in heterotopic tumor tissues and plasma. Drug accumulation was measured in both plasma and tumor samples ($n = 4$ mice per group). Tumors were homogenized in ice-cold PBS and quantitative analysis was carried out using a Waters Quattro microtriple quad liquid chromatography

coupled mass spectroscopy system with a Phenomenex Gemini-NX C18 as per the manufacturer's instructions.

Orthotopic pancreatic cancer model. Panc-1 pancreatic cancer cells were stably transduced with luciferase and the orthotopic model was established as previously described (19).

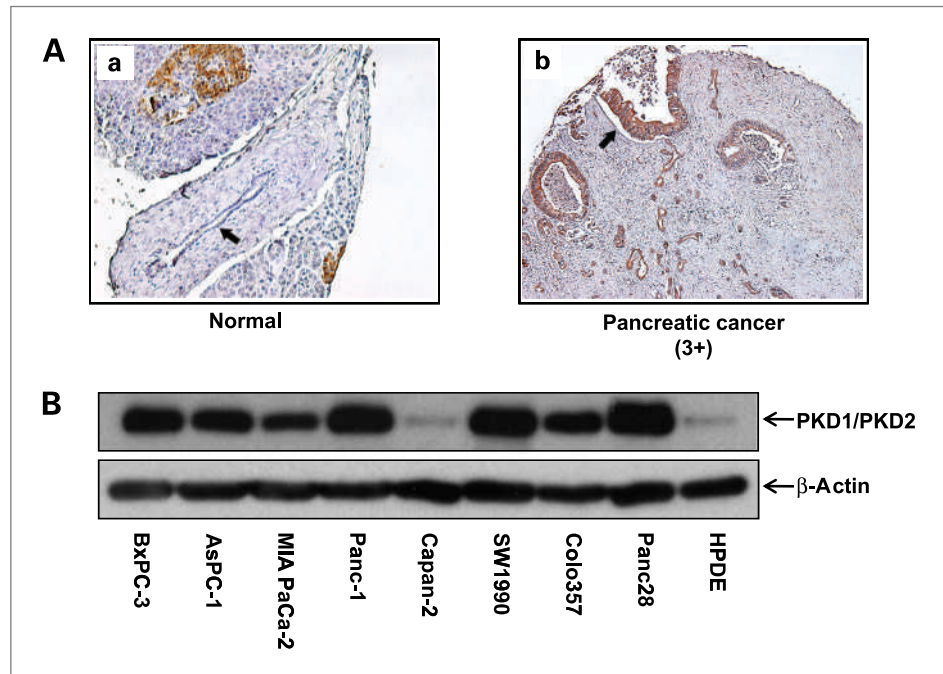
Experimental protocol for orthotopic pancreatic cancer model and bioluminescence imaging. One week after implantation of Panc-1 cells, mice were randomized into the following groups ($n = 7$ mice per group) based on the bioluminescence measured after the first IVIS imaging: (a) nontreated control (vehicle; 5% dextrose orally) and (b) CRT0066101 (80 mg/kg dissolved in 5% dextrose) given orally (by gavage) once daily. Tumor volumes were monitored weekly by the bioluminescence IVIS Imaging System 200 as previously described (19). Therapy was given for 3 weeks and animals were sacrificed on day 35 after tumor implantation. Primary tumors in the pancreas were excised and the final tumor volume was measured as $V = 2/3\pi r^3$, in which r is the mean of the three dimensions (length, width, and depth). The final tumor volumes were initially subjected to one-way ANOVA and then later compared among groups using unpaired Student's *t* test. Half of the tumor tissue was fixed with formalin and paraffin embedded for immunohistochemistry (IHC) and routine H&E staining. The other half was snap frozen in liquid nitrogen and stored at -80°C . H&E staining confirmed the presence of tumor in each group.

Western blots. Pancreatic tumor tissues (75–100 mg/mouse; $n = 2$ per group) from control and CRT0066101-treated mice were minced, homogenized using a Dounce homogenizer, centrifuged at $16,000 \times g$ at 4°C for 10 minutes, and treated with ice-cold lysis buffer for the preparation of cellular lysates. Similarly, whole-cell lysates were prepared from indicated pancreatic cancer and HPDE cell line as mentioned. Whole-cell lysates or cytoplasmic lysates (CE) were prepared from Panc-1 cells either transfected with control (Panc-1) or PKD1-overexpressing vectors (Panc-1-PKD1) using ice-cold lysis buffer as mentioned. The proteins were then fractionated by SDS-PAGE, electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Ki-67 analysis. Formalin-fixed and paraffin-embedded sections (5 μ m) were stained with Ki-67 (rabbit monoclonal clone SP6, NeoMarkers) antibody (20). Results were expressed as percent of Ki-67-positive cells \pm SEM per $\times 200$ magnification. A total of 10 high-power fields (HPF) were examined and counted from each treatment groups ($n = 7$ per group). The values were compared both using unpaired Student's *t* test and Fisher's exact test with $P < 0.05$.

In situ TUNEL assay. Cryostat sections (5 μ m) were fixed in 4% paraformaldehyde [in PBS (pH 7.4)] and *in situ* TUNEL assay (Roche Diagnostics) was done as per the manufacturer's instructions previously described

Figure 1. Increase of activated PKD1/2 in pancreatic ductal adenocarcinoma (pancreatic cancer) and PKD family expression in pancreatic cancer cell lines. A, representative IHC photomicrographs of activated PKD1/2 in pancreatic cancer tissue microarrays depicting normal pancreatic ducts and pancreatic cancer as described in Materials and Methods. B, Western blot analysis of PKD1/2 in multiple human pancreatic cancer cell lines and in immortalized HPDE cells as described in Materials and Methods.



(20). A total of five HPFs ($\times 400$) were examined and counted for TUNEL-positive and total number of cells from each treatment groups ($n = 7$ per group). Results were expressed as the percent of TUNEL-positive cells \pm SEM per $\times 400$ magnification (HPF). The values were compared both using unpaired Student's *t* test and Fisher's exact test with $P < 0.05$.

Microvessel density. Cryostat sections ($5 \mu\text{m}$) were stained with rat anti-mouse CD31 monoclonal antibody (BD Biosciences). Areas of greatest vessel density were then examined under higher magnification ($\times 200$) and counted (20). Results were expressed as the mean number of vessels \pm SEM per HPF. A total of 10 HPFs were examined and counted from each treatment group ($n = 7$ per group). The values were compared both using unpaired Student's *t* test and Fisher's exact test with $P < 0.05$.

Results

Active and total PKD family expression in pancreatic cancer. We showed for the first time the autophosphorylated (active) PKD family expression by IHC analysis of a human pancreatic cancer tissue microarray (Fig. 1A). The monoclonal antibody used for IHC detects autophosphorylated PKD1 and PKD2 on Ser-910 and Ser-876, respectively. PKD3 does not contain a residue that can be phosphorylated at the equivalent COOH-terminal position. As shown in Table 1, active PKD1/2 was significantly expressed in pancreatic cancer tissues compared with normal ducts (91% versus 22%). The intensity of IHC staining was independently graded by a pathologist, who was blinded to this study. Representative photomicrographs

of IHC staining intensity are shown in Fig. 1A and Supplementary Fig. S1A. Furthermore, the majority of pancreatic cancer tissues showed increased IHC staining intensity compared with normal ducts (Supplementary Fig. S1B). We also examined expression of total PKD1/2 in multiple pancreatic cancer cell lines and in immortalized HPDE cells. The antibody used (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) recognizes both, PKD1 and PKD2 (but not PKD3). As shown in Fig. 1B, most of the pancreatic cancer cell lines examined exhibited significant expression of PKD1/2 in comparison with HPDE.

Discovery of CRT0066101 as a PKD-specific small-molecule inhibitor. To validate PKD as a potential anticancer target, we screened a diverse compound library against purified PKD family enzymes to identify novel inhibitors against this protein kinase family. The backbone structure of the novel PKD inhibitors is depicted in Fig. 2A. A lead member of this family, CRT0066101,

Table 1. Summary of activated PKD1/2 IHC grading in pancreatic cancer tissue microarrays as described in Materials and Methods

	pS916-PKD1/2		Total
	Negative (-)	Positive (+)	
Normal pancreatic ducts	14 (78%)	4 (22%)	18
Pancreatic ductal adenocarcinoma	5 (9%)	46* (91%)	51

* $P < 0.0001$ (Fisher's test).

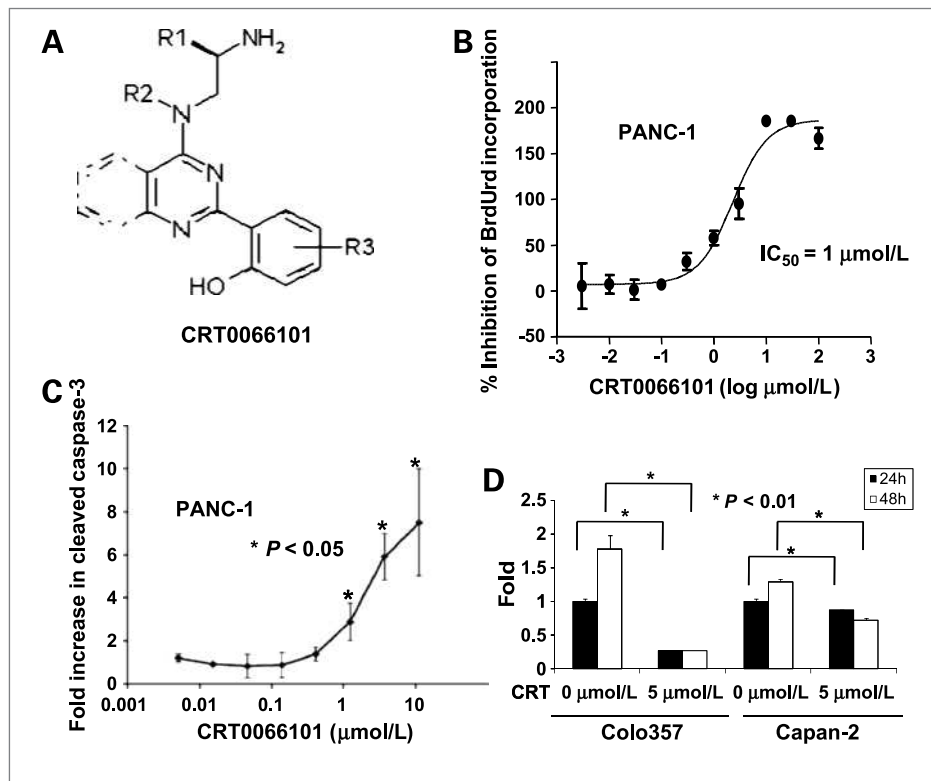


Figure 2. Effects of CRT0066101 on Panc-1 cells *in vitro*. **A**, chemical structure of a typical amino-ethyl-aryl compound, in which *R1*, *R2*, and *R3* represent functional groups. This represents the backbone structure of CRT0066101 and its integrity confirmed by liquid chromatography coupled mass spectrometry and nuclear magnetic resonance. **B**, Panc-1 cells were treated with CRT0066101 before stimulation with 20% serum and BrdUrd incorporation was measured by ArrayScan as described in Materials and Methods. CRT0066101 inhibited both serum-stimulated and basal Panc-1 proliferation (as measured by BrdUrd incorporation), thereby leading to 200% inhibition at the maximal dose tested. **C**, Panc-1 cells were serum starved for 6 h and levels of cleaved caspase-3 were determined using the Meso Scale Discovery multiplex apoptosis panel kit as described in Materials and Methods. Points mean with $P < 0.05$ compared with 0.5 $\mu\text{mol/L}$; bars, SEM. **D**, Colo357 and Capan-2 cells were either treated with control vehicle DMSO (0 $\mu\text{mol/L}$) or CRT0066101 (5 $\mu\text{mol/L}$) for 24 and 48 hours, respectively. Cell proliferation was determined by using the CellTiter Aqueous One Solution Cell Proliferation Assay kit as described in Materials and Methods. All the experiments were done in triplicate. Columns, mean and is presented as fold change over nontreated control (24 h) with $P < 0.01$; bars, SEM.

was used *in vitro* to quantitate its effects on the catalytic activity of PKD family members, as determined by the inhibition of peptide substrate phosphorylation. The biochemical IC_{50} values were 1, 2.5, and 2 nmol/L for PKD1, 2, and 3, respectively (data not shown). The specificity of CRT0066101 for PKD family members was also confirmed in an *in vitro* kinase assay comprising a panel of >90 protein kinases including PKC α /PKB α /mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase/c-Raf/c-Src/c-Abl that have a role in cancer promotion or progression. Using intact Panc-1 cells as our model system and a high-throughput fast activated cell-based ELISA assay, the IC_{50} value was 0.5 $\mu\text{mol/L}$ (Supplementary Fig. S2A). CRT0066101 specifically blocked PKD1/2 activity and did not suppress PKC α /PKC β /PKC ϵ activity in multiple cancer cell types including A549 (lung) and MiaPaCa-2 (pancreas).

CRT0066101 reduced proliferation, increased apoptosis, and reduced the cell viability of pancreatic cancer cells. Based on our results in Fig. 1B, we have used Panc-1 cells

expressing moderate levels of PKD1/2 as our model system. As shown in Fig. 2B, CRT0066101 significantly inhibited Panc-1 cell proliferation, as revealed by inhibition of BrdUrd incorporation, with an IC_{50} value of 1 $\mu\text{mol/L}$. Treatment with CRT0066101 resulted in a 6- to 10-fold induction of apoptosis in Panc-1 cells, as judged by the levels of cleaved caspase-3 (Fig. 2C). Next, we evaluated the sensitivity to CRT0066101 of pancreatic cancer cells with varying endogenous levels of PKD1/2 expression. Specifically, we measured the cell proliferation of pancreatic cancer cells either expressing moderate-high (Colo357, Panc-1, MiaPaCa-2, and AsPC-1) or low (Capan-2) levels of PKD1/2 after treatment without or with CRT0066101 (5 $\mu\text{mol/L}$) for 24 and 48 hours. As shown in Fig. 2D and Supplementary Fig. S2B, CRT0066101 significantly reduced the cell proliferation of Colo357, Panc-1, MiaPaCa-2, and AsPC-1 cells but had a modest effect in Capan-2 cells. These results suggest that CRT0066101 significantly blocked the proliferation of pancreatic cancer cells that express moderate to high endogenous levels of PKD1/2.

CRT0066101 reduced the agonist-induced PKD activation and PKD-dependent phosphorylation of Hsp27 in Panc-1. To determine the specificity of CRT0066101 inhibition of PKD1/2 activity induced by a physiologic agonist

through receptor-mediated pathways, we examined whether it prevents PKC-dependent PKD1/2 activation induced by the G protein-coupled receptor (GPCR) agonist neurotensin in pancreatic cancer cells. As shown

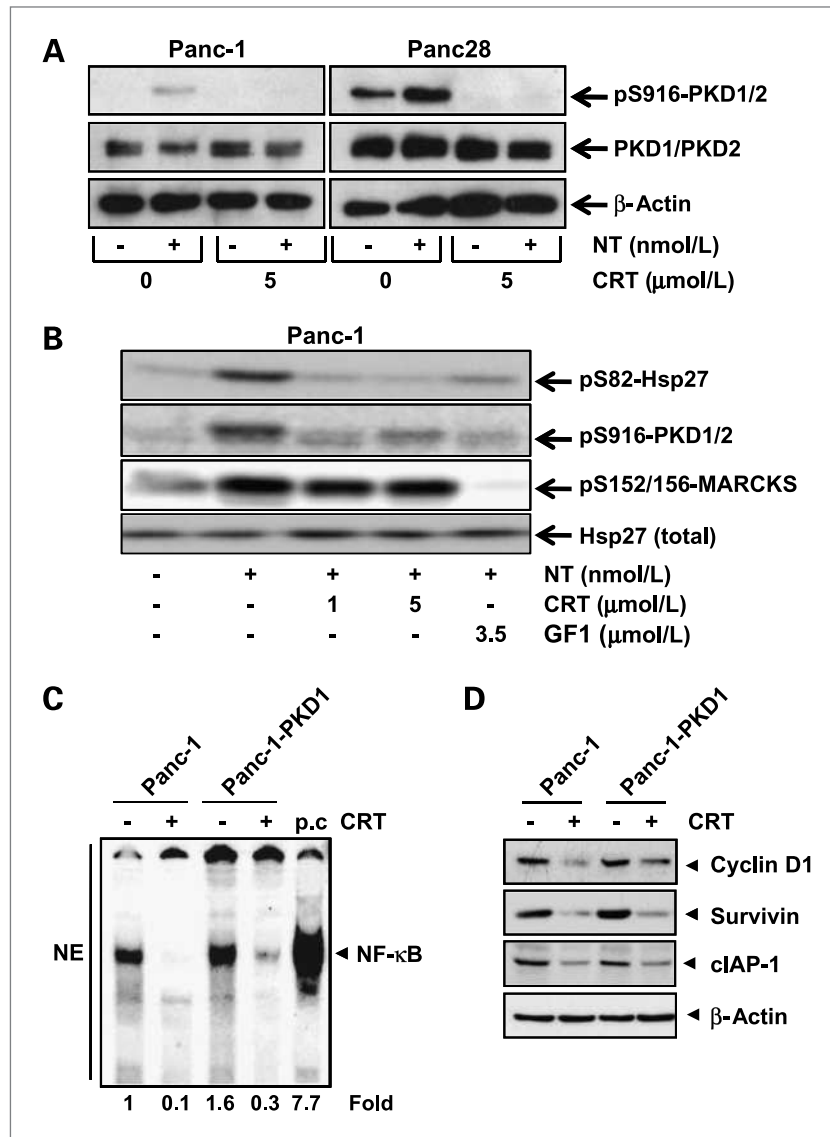


Figure 3. Effects of CRT0066101 on agonist-mediated PKD activation, PKD-mediated substrate phosphorylation, NF-κB activation, and expression of NF-κB-dependent gene products in pancreatic cancer cells. **A**, serum-starved cultures of Panc-1 and Panc-28 cells were treated either with control vehicle DMSO (0 μmol/L) or CRT0066101 (5 μmol/L) for 1 h before stimulation without (-) or with 50 nmol/L of neurotensin for 10 min. Cell lysates were prepared and Western blots were performed with antibodies against pS916-PKD1/2 and total PKD1/2 as described in Materials and Methods. The membrane was reprobbed with β-actin to verify equal loading. **B**, serum-starved cultures of Panc-1 cells were incubated in the absence (-) or in the presence of 5 or 1 μmol/L CRT0066101 or 3.5 μmol/L GF 109203X (GF1) for 1 h before stimulation without (-) or with 5 nmol/L neurotensin for 10 min. Cell lysates were prepared and Western blots were done with antibodies against pS82-Hsp27, pS916-PKD1/2, and pS152/156-MARCKS. The membrane was reprobbed with total Hsp27 antibody to verify equal loading. **C**, Panc-1 cells were either transfected with control (Panc-1) or PKD1-overexpressing (Panc-1-PKD1) vectors and 23 h posttransfection were treated with DMSO (-) or 5 μmol/L CRT0066101 (+) for 1 h before preparation of nuclear extracts for measuring NF-κB activation by EMSA as described in Materials and Methods; p.c., positive control (nuclear extracts from KBM-5 cells stimulated with tumor necrosis factor-α). Results in the bottom of EMSA are expressed as fold activity of the nontreated control (Panc-1 cells transfected with mock vector). **D**, Panc-1 cells were either transfected with control (Panc-1) or PKD1-overexpressing (Panc-1-PKD1) vectors and 23 h posttransfection were treated with DMSO (-) or 5 μmol/L CRT0066101 (+) for 1 h before preparation of whole-cell lysates to analyze the effects on NF-κB-dependent gene expression. Western blots were then performed with antibodies against cyclin D1, survivin, and cIAP-1 as described in Materials and Methods. The membrane was reprobbed with β-actin to verify equal loading. All the results shown are representatives of three independent experiments.

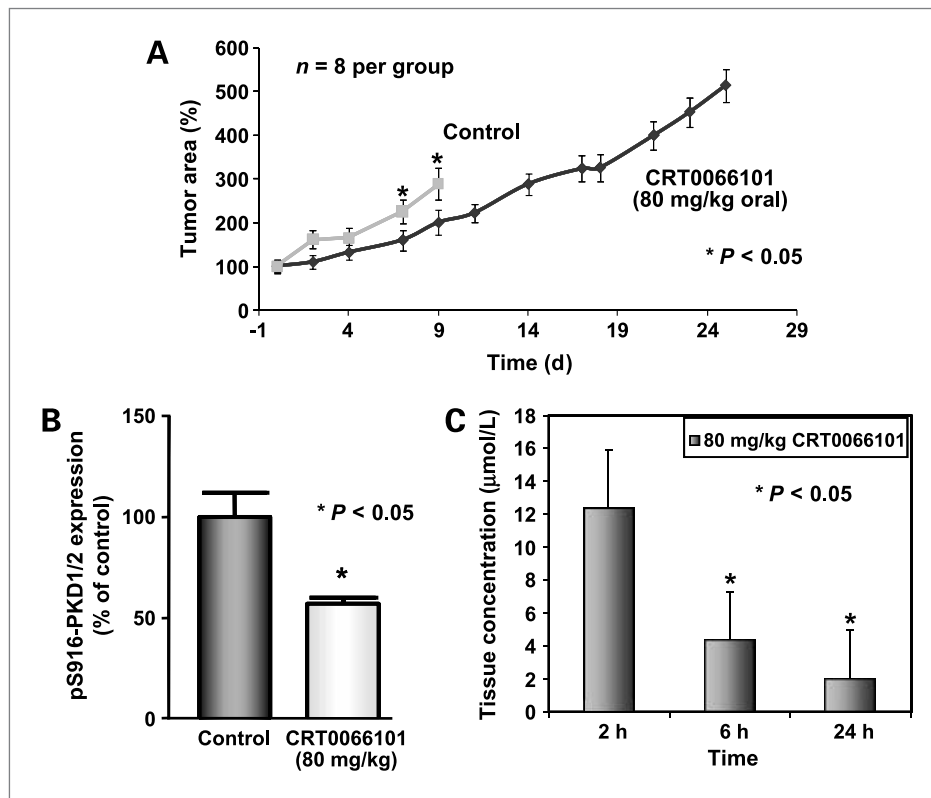


Figure 4. Effects of oral administration of CRT0066101 *in vivo* (heterotopic Panc-1 xenograft model). **A**, mean tumor area depicted as tumor area (percent or %) of Panc-1 heterotopic xenografts in *nu/nu* mice following treatment with control (5% dextrose) or CRT0066101 ($n = 8$) as described in Materials and Methods. All the mice in the control group were sacrificed at day 9 as the mean tumor area reached the cutoff value ($>1.44 \text{ cm}^2$) per CRUK Institutional Animal Care and Use Committee guidelines. Points, mean; bars, SEM. **B**, quantified expression of activated PKD1/2 in Panc-1 tumors ($n = 4$ per group) 2 h after last treatment with CRT0066101 as described in Materials and Methods. Columns, mean with (*) $P < 0.05$ versus control; bars, SEM. **C**, tumor concentrations of CRT0066101 ($n = 4$) as described in Materials and Methods. Samples were collected 2, 6, and 24 h after mice received their 5th dose. In **A** and **B/C**, *nu/nu* mice were given CRT0066101 (80 mg/kg, once daily, oral) for 24 or 5 d, respectively. Columns, mean with (*) $P < 0.05$ versus 2-h tissue concentration; bars, SEM.

in Fig. 3A, CRT0066101 (5 $\mu\text{mol/L}$) blocked both the basal and neurotensin-induced pS916-PKD1/2 (activated PKD1/2) in Panc-1 and Panc-28 cells. Next, we showed that CRT0066101 in Panc-1 cells abrogated the neurotensin-induced phosphorylation of Hsp27 (pS82-Hsp27), which is a physiologic substrate of PKD1/2 (12), in a dose-dependent manner (Fig. 3B). Interestingly, CRT0066101 did not block phosphorylation of myristoylated alanine-rich C kinase substrate (pS152/156-MARCKS), which is a known PKC substrate (Fig. 3B). In contrast, GF-1, a conventional and novel PKC isoform-selective inhibitor, potently blocked the expression of pS152/156-MARCKS, pS916-PKD1/2, and pS82-Hsp27 (Fig. 3B). These results strongly suggest that CRT0066101 is a PKD-specific inhibitor.

CRT0066101 reduced the PKD-dependent NF- κ B activation and NF- κ B-dependent gene expressions in Panc-1. To further dissect the mechanism by which PKD1/2 enhances cell proliferation and prosurvival signaling, we investigated the effect of its inhibition on NF- κ B, a pivotal transcription factor that regulates these biological processes (21–23). We and others have shown

that GPCR-mediated signaling through PKD1/2 regulates NF- κ B gene products in multiple cell types (4, 24, 25). Specifically, we examined whether CRT0066101 could block PKD1/2 overexpression-induced NF- κ B activity in pancreatic cancer cells. Using Panc-1 cells as our model system, we showed that PKD1 overexpression induced NF- κ B activity by 60% and this effect was blunted by CRT0066101 (5 $\mu\text{mol/L}$), as shown by the EMSA of nuclear extracts in Fig. 3C. In agreement with previous reports (19), we observed a constitutive NF- κ B activation in control-vector-transfected Panc-1 cells. As noted in Fig. 1B, Panc-1 cells express moderate levels of PKD1/2 that can partly explain constitutive NF- κ B activation in these cells. In addition, ligand-induced physiologic activation of PKD1/2 potently stimulated NF- κ B-dependent reporter gene activation over the constitutive basal level. As shown in Supplementary Fig. S3A, CRT0066101 reduced tumor necrosis factor- α -induced NF- κ B-dependent reporter gene (luciferase) activity in Panc-1 cells in a dose-dependent manner at 6 and 24 hours after stimulation. Similar results were obtained in pancreatic cancer cells including AsPC-1 and Panc-28. Furthermore, we

showed that CRT0066101 significantly inhibited PKD2-mediated NF- κ B-dependent reporter gene (luciferase) activity (Supplementary Fig. S3B). Similar results were obtained with PKD1 overexpression in Panc-1 cells. To confirm that CRT0066101 abrogated NF- κ B-dependent gene products that are critical for cell proliferation and survival (21–23), we showed that CRT0066101 potently inhibited the expression of cyclin D1, survivin, and cIAP-1 in both Panc-1 and Panc-1-PKD1-overexpressing cells (Fig. 3D). Interestingly, we observed an increased expression of survivin after PKD1 overexpression in Panc-1 cells that was significantly attenuated by CRT0066101. Thus, our results suggest that PKD1/2 promotes the activation of NF- κ B and its gene products.

CRT0066101 inhibited the growth of tumors in a subcutaneous pancreatic cancer model. Pharmacokinetic parameters were determined after a single bolus dose of CRT0066101. The terminal half-life and bioavailability were determined to be 60 minutes and ~100%, respectively (data not shown). Furthermore, plasma concentrations of CRT0066101 were evaluated following administration of daily oral doses of 80 mg/kg for 5 days in CD-1 mice, and as shown in Supplementary Fig. S4A, optimal therapeutic concentrations (8 μ mol/L) of CRT0066101 were detectable 6 hours after oral adminis-

tration of this drug. These results prompted us to further evaluate detailed pharmacologic properties of CRT0066101 *in vivo*. We treated established tumors in subcutaneous Panc-1 xenograft models with a dose of 80 mg/kg CRT0066101, given orally once daily, or vehicle control. There were no signs of toxicity throughout the treatment period as shown by stable bodyweights during the entire treatment period (Supplementary Fig. S4B). We observed a statistically significant reduction (by 48.5%) in tumor volume at day 9 in the treated group (Fig. 4A). To substantiate that the effects on tumor growth were mediated through the inhibition of PKD activity, PKD1/2 autophosphorylation was measured in subcutaneous Panc-1 tumor explants following treatment with five oral doses of CRT0066101 or vehicle control. We observed a statistically significant but modest reduction of PKD1/2 autophosphorylation 2 hours after the cessation of dosing (Supplementary Fig. S4C; Fig. 4B) and this effect was not quantifiable at subsequent time points (data not shown). Concentrations above those required to elicit effects *in vitro* were detectable in plasma (data not shown) and in Panc-1 tumor explants up to 6 hours following this treatment regimen (Fig. 4C).

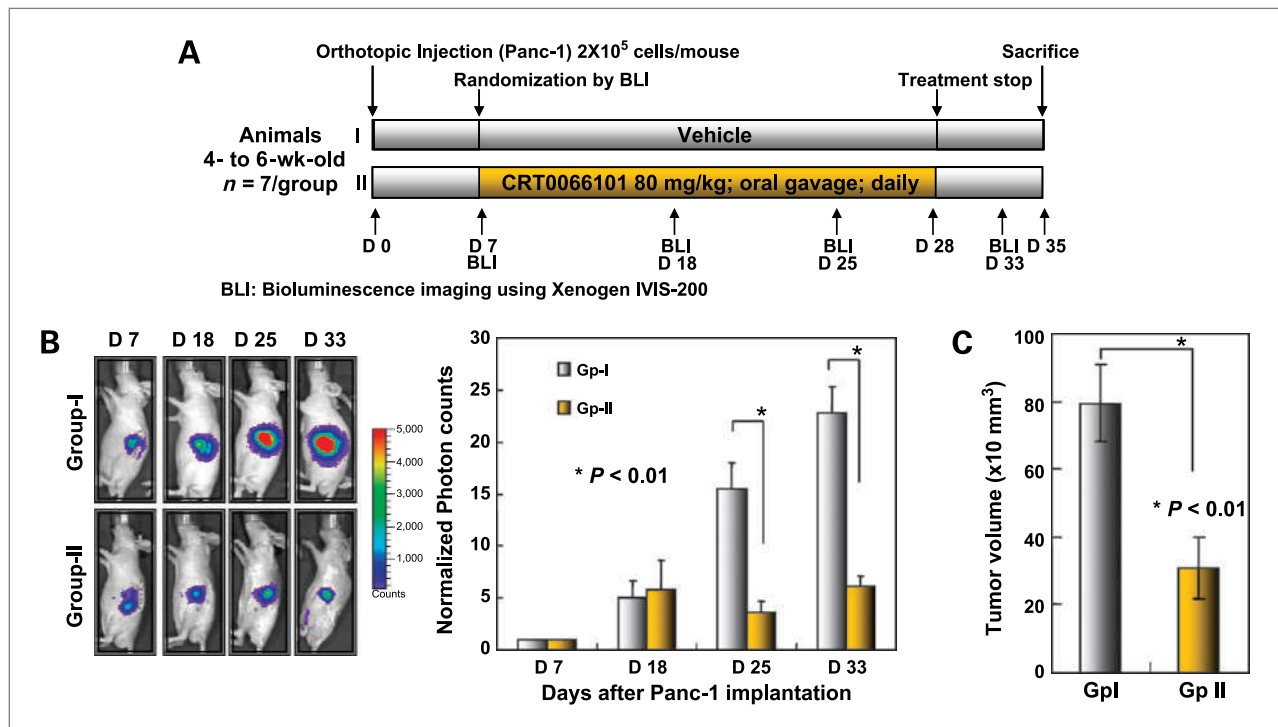


Figure 5. Effects of oral administration of CRT0066101 in an orthotopic Panc-1 xenograft model. **A**, schematic representation of experimental protocol. Groups I and II were treated with vehicle (5% dextrose) or CRT0066101 (80 mg/kg, once daily, oral, $n = 7$), respectively. **B**, left, IVIS bioluminescence images of orthotopically implanted pancreatic tumors in live anesthetized mice as described in Materials and Methods. **B**, right, measurements of photons per second depicting tumor volumes of Gp I (white) and Gp II (yellow) using live IVIS imaging were plotted at the indicated times ($n = 7$) as described in Materials and Methods. Columns, mean; bars, SEM. *, $P < 0.01$. **C**, Gp I (white) and Gp II (yellow) in each mouse measured during autopsy using Vernier calipers and calculated using the formula $V = 2/3r^3$ ($n = 7$) as described in Materials and Methods. Columns, mean; bars, SEM. *, $P < 0.01$.

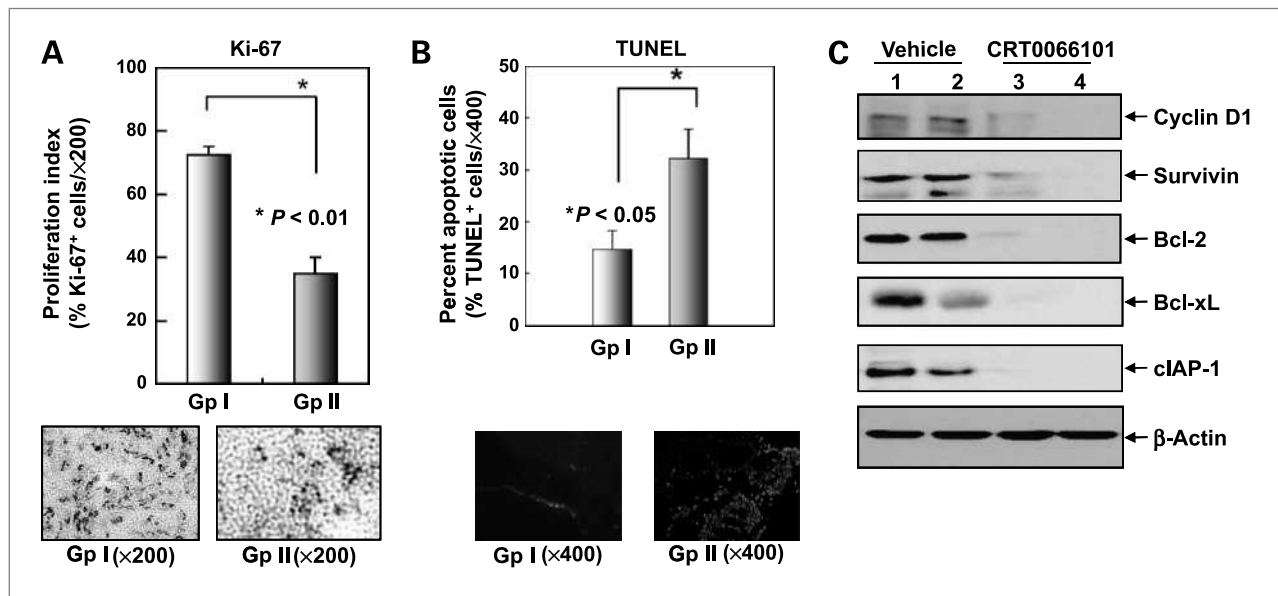


Figure 6. Effects of CRT0066101 on proliferation, apoptosis, proliferative, and prosurvival gene products in orthotopic pancreatic cancer tumor explants. A, quantification of Ki-67⁺ cells or proliferation index as described in Materials and Methods. Columns, means ($n = 7$); bars, SEM. *, $P < 0.01$. Representative Ki-67⁺ IHC are shown for group I or (Gp I; control) and group II (Gp II; CRT0066101) at $\times 200$. B, quantification of *in situ* TUNEL-positive cells as described in Materials and Methods. Columns, means ($n = 7$); bars, SEM. *, $P < 0.05$. Representative TUNEL-positive cells are shown for group I (control) and group II CRT0066101) at $\times 400$. C, Western blot analysis of proliferative (cyclin D1) and prosurvival proteins (survivin, Bcl-2, Bcl-xL, and cIAP-1) in cellular lysates from group I (control) and group II (CRT0066101) tumor explants as described in Materials and Methods. Cell lysates were prepared from each tumor explants obtained from two distinct animals in vehicle (1 and 2) and CRT0066101-treated (3 and 4) groups. The membrane was probed with β -actin to verify equal loading.

CRT0066101 reduced growth of tumors in an orthotopic pancreatic cancer model. The effects of CRT0066101 in orthotopically implanted Panc-1 cells in nude mice were investigated as shown in Fig. 5A (26). Bioluminescence was measured as a surrogate marker to assess tumor volume. The bioluminescence imaging (Fig. 5B, left) indicated that there is an increase of tumor volume in the control group compared with the treated group. The normalized photon counts showed statistically significant reduction after 18 days of treatment with CRT0066101 and continued for 5 days beyond the cessation of treatment (Fig. 5B, right). As shown in Fig. 5C, there was a 58% reduction of final tumor volume at the end of trial in the treated group.

To understand the mechanism of reduction in tumor volume by CRT0066101, we examined for proliferation and apoptosis markers in these tumor explants. Ki-67 (proliferation marker) expression was significantly reduced (by 52%) in the treated group (Fig. 6A) and the *in situ* TUNEL assay showed 58% increased apoptosis in tumor explants of the treated group (Fig. 6B) compared with the control. CRT0066101 inhibited expression of cyclin D1 (Fig. 6C), which is involved in tumor cell proliferation (27). Overexpression of NF- κ B-dependent gene products including survivin, Bcl-2, Bcl-xL, and IAP-1 is linked to tumor survival, chemoresistance, and radioreistance (28). CRT0066101 treatment abrogated the expression of these proteins in tumor explants (Fig. 6C). We also examined the effects of CRT0066101 on tumor-

associated angiogenesis as this process is critical for tumor survival and proliferation (2, 20). CRT0066101 treatment reduced CD31⁺ microvessel density by 60% in the tumor explants (Supplementary Fig. S5A) and abrogated the expression of cyclooxygenase-2 and vascular endothelial growth factor (Supplementary Fig. S5B), important mediators of pancreatic cancer-associated angiogenesis.

Discussion

The PKD family regulates multiple key cellular functions and is implicated in the pathogenesis of neoplastic disorders, including pancreatic cancer (29). One of the salient features presented here is that activated PKD1/2 is significantly upregulated in pancreatic cancer compared with normal pancreatic ducts. IHC of a pancreatic cancer tissue microarray showed strongly positive staining in the majority of pancreatic cancer neoplastic epithelia, whereas most of the normal ductal epithelia had negative or minimal staining.

Despite the evidence supporting the fundamental role of PKD in cancer and consequentially as a putative target, there has been limited progress in the development of selective inhibitors. Such inhibitors are required for detailed pharmacologic investigations of inhibition of the molecular target. Consequently, we established a PKD-specific small-molecule discovery program and identified a prototype compound CRT0059359 with activity *in vitro*. To obtain a bioavailable pharmacologically

active compound, medicinal chemistry and biological optimization were used to identify CRT0066101 as our lead product. Our *in vitro* studies noticeably showed that CRT0066101 is a PKD-specific inhibitor as it potently blocked GPCR agonist-induced PKD activation, abrogated the PKD-specific phosphorylation of a physiologic substrate (Hsp27) involved in chemoresistance, and reduced PKD-mediated NF- κ B activation in pancreatic cancer cells. Indeed, CRT0066101-mediated inhibition of PKD1/2 decreased NF- κ B activity and the expression of NF- κ B-dependent gene products essential for cell proliferation and survival in pancreatic cancer cells. In two distinct murine pancreatic cancer models, CRT0066101 significantly abrogated tumor growth without any detectable systemic toxicity and increased survival period (a surrogate marker) in the subcutaneous model. Further, CRT0066101 reduced proliferation, increased apoptosis, and reduced angiogenesis in our orthotopic pancreatic cancer model.

There are several reports of PKD inhibitors in the literature including Go6976 (30), H-89 (31), and the polyphenolic cancer chemopreventive agent *trans*-3-4',5-trihydroxystilbene (resveratrol; ref. 32). However, the therapeutic potential of these agents may be hindered by their poor selectivity or high concentrations that would be required to achieve pharmacologic effects (32). None of these compounds have been tested as therapeutic agents *in vivo*. Recently, a noncompetitive selective PKD inhibitor CID755673 was identified from the NIH repository of small molecules (33). CID755673 significantly reduced proliferative, migratory, and invasive phenotypes in prostate cancer cells with IC₅₀ values between 10 and 30 μ mol/L (33) but also induced unexpected mitogenic effects (34). It will be important to establish the therapeutic potency and toxicity profile of CID755673 *in vivo*. CRT0066101 is struc-

turally distinct from CID755673 and inhibits the activity of all PKD isoforms (PKD1, PKD2, and PKD3). More importantly, CRT0066101 has significant activities *in vitro* in the range of 0.5 to 5 μ mol/L and we show the feasibility of achieving these concentrations of drug in mice following oral dosing.

In conclusion, this study identifies CRT0066101 as a potent inhibitor of the PKD family and validates the role of PKD1/2 in pancreatic cancer tumorigenesis. We showed, *for the first time*, that this inhibitor was orally bioavailable and blocked tumor growth *in vivo*. Our data support further evaluation of this series of novel anticancer therapeutics.

Disclosure of Potential Conflicts of Interest

Cancer Research Technologies owns a patent relating to research in this article. No other potential conflicts of interest were disclosed.

Acknowledgments

We would like to dedicate this manuscript to the memory of Professor Lloyd Kelland, who unfortunately passed away during the preparation of this manuscript.

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Correction: A Novel Small-Molecule Inhibitor of Protein Kinase D Blocks Pancreatic Cancer Growth *In vitro* and *In vivo*

In this article (Mol Cancer Ther 2010;9:1136–46), which was published in the May 1, 2010 issue of *Molecular Cancer Therapeutics* (1), one of the authors' name was misspelled. The correct spelling of the name is Azadeh Bagherzadeh. The online article has been changed to reflect this correction and no longer matches the print.

Reference

1. Harikumar KB, Kunnumakkara AB, Ochi N, et al. A novel small-molecule inhibitor of protein kinase D blocks pancreatic cancer growth *in vitro* and *in vivo*. Mol Cancer Ther 2010;9:1136–46.

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