Protein kinase Cδ inactivation inhibits cellular proliferation and decreases survival in human neuroendocrine tumors

Zhihong Chen1, Lora W Forman1, Kenneth A Miller3, Brandon English3, Asami Takashima1, Regine A Bohacek4, Robert M Williams3 and Douglas V Faller1,2

1Cancer Center, K-701 and 2Departments of Medicine, Pediatrics, Biochemistry, Microbiology, Pathology and Laboratory Medicine, Boston University School of Medicine, 72 East Concord Street, Boston, Massachusetts 02118, USA
3Department of Chemistry, Colorado State University, 115 Centre Street, Fort Collins, Colorado, USA
4Boston De Novo Design, Boston, Massachusetts, USA
(Correspondence should be addressed to D V Faller at Cancer Center, K-701, Boston University School of Medicine; Email: dfaller@bu.edu)

Abstract

The concept of targeting cancer therapeutics toward specific mutations or abnormalities in tumor cells, which are not found in normal tissues, has the potential advantages of high selectivity for the tumor and correspondingly low secondary toxicities. Many human malignancies display activating mutations in the Ras family of signal-transducing genes or over-activity of p21Ras-signaling pathways. Carcinoid and other neuroendocrine tumors have been similarly demonstrated to have activation of Ras signaling directly by mutations in Ras, indirectly by loss of Ras-regulatory proteins, or via constitutive activation of upstream or downstream effector pathways of Ras, such as growth factor receptors or PI3-kinase and Raf/mitogen-activated protein kinases. We previously reported that aberrant activation of Ras results in an absolute dependency on PKCδ-mediated survival pathways (Xia et al. 2007, 2009). Over-activity of Ras signaling, therefore, sensitizes tumor cells to apoptosis induced by suppression of PKCδ activity, whereas suppression of PKCδ activity is not toxic to cells with normal levels of Ras activity or signaling (Chen & Faller 1995, 1996, Chen et al. 1998a, b, 2001, 2003, Liou et al. 2000, 2004, Xia et al. 2007). We have shown that this tumor-specific susceptibility,
designated ‘Ras-mediated apoptosis’, can be exploited as a targeted cancer therapeutic.

Bronchopulmonary, gastrointestinal, and pancreatic neuroendocrine tumors are rare tumors originating from neuroendocrine tissues (Oberg 1999). Clinical symptoms are often caused by the production of hormonally active substances by the tumor such as serotonin, gastrin, insulin, vasoactive intestinal peptide, pancreatic polypeptide, or substance P. Chromogranin A is produced by 80–100% of neuroendocrine tumors and serves as a reliable biochemical marker. The disease can be cured by early surgery, but the vast majority of tumors have metastases at the time of diagnosis, which makes palliation the cornerstone of management. Debulking surgery, liver artery embolization, and chemotherapy aim at tumor mass reduction, whereas somatostatin analogs and IFN are used for control of symptoms (Frank et al. 1999, Arnold et al. 2000). Radioactively labeled somatostatin analogs have been used in trials, with response rates ~30% (Arnold et al. 2002). Response rates of cytoreductive approaches are generally below 60%, however, and long-term responses are not maintained (Oberg 2001). New and more effective approaches are, therefore, needed in the treatment of neuroendocrine malignancies.

Carcinoid and other neuroendocrine tumors of the gastrointestinal tract share a number of same genetic abnormalities (deletions and mutations) as adenocarcinomas (Arber et al. 1997, Leotlela et al. 2003). These abnormalities include activation of Ras signaling directly by mutations in the Ras protein, indirectly by loss of Ras-regulatory proteins such as NF-1, or via constitutive activation of Ras-linked growth factor receptors, or downstream effector pathways of Ras, such as PI3K and Raf/mitogen-activated protein kinases (Raf/MAP kinases). For example, activation of H-Ras and Ki-Ras signaling is detected in a significant fraction of carcinoid and other gastrointestinal neuroendocrine tumors (65 and 10% respectively; Liedke et al. 1998, Maitra et al. 2000). Ras itself can be activated in neuroendocrine tumors by point mutation or by loss of regulators of Ras, such as RassF1A or NF-1 (Stancu et al. 2003, Liu et al. 2005, Bausch et al. 2007). The Raf/MAP kinase, or the MAP kinases directly downstream of Raf, is frequently activated in neuroendocrine tumors (Perren et al. 2004, Tannapfel et al. 2005, Kunnimalaiyaan & Chen 2006, Karhoff et al. 2007). The PI3K pathway can be activated in neuroendocrine tumors from deletion of the tumor suppressor gene phosphatase and tensin homolog (PTEN). Loss of PTEN in neuroendocrine tumors increases in frequency with the loss of differentiation in the tumor (Wang et al. 2005), and loss of PTEN expression may represent an important step in the progression of neuroendocrine tumors (Wang et al. 2002).

We demonstrate in this report that human neuroendocrine tumor cell lines of pulmonary and gastrointestinal origin are sensitive to PKCδ inhibition. Knockdown of PKCδ by exposure to PKCδ-specific shRNA, or suppression of PKCδ activity by diverse small-molecule inhibitors, is sufficient to inhibit proliferation of these human neuroendocrine tumor cell lines and efficiently induce apoptosis.

Materials and methods

Cell lines

BON1, a human foregut (pancreatic) carcinoid tumor cell line (Parekh et al. 1994), was obtained from Kjell Oberg (Uppsala University, Sweden) through Dr Evan Vosburgh. H727 cells, derived from a human bronchopulmonary carcinoid tumor (Schuller et al. 1987), were purchased from ATCC (Manassas, VA, USA). The CNDT 2.5 cell line, initially described as a human midgut carcinoid tumor cell line (Van Buren et al. 2007), was provided by Dr Lee Ellis (MD Anderson Cancer Center). The provenance of this cell line is currently under review by the originator. NIH-3T3 and NIH-Ras cells have been previously described (Xia et al. 2007, 2009). MCF10, BxPC3, and PZ-HPV-7 cells were obtained from ATCC. BON1, H727, and CNDT 2.5 cells were propagated in 10% fetal bovine serum (Invitrogen), DMEM/Hams F-12 50:50 media (Cellgro, Mediatech, Manassas, VA, USA), 2 mM-glutamine (Invitrogen, Carlsbad, CA, USA), 200 U penicillin/ml; 200 μg streptomycin/ml (Invitrogen); 10 ng/ml nerve growth factor (Invitrogen); 1× MEM non-essential amino acids (Cellgro); 1× MEM vitamin solution (Cellgro); 1 mM sodium pyruvate; and 0.015 M HEPES buffer (pH 7.3; American Bioanalytical, Natick, MA, USA). NIH-3T3, NIH-Ras, MCF10, MCF1-Ras, BxPC3, and PZ-HPV-7 cells were propagated in 10% fetal bovine serum (Invitrogen), DMEM (Cellgro), 2 mM l-glutamine (Invitrogen); 200 U penicillin/ml; and 200 μg streptomycin/ml (Invitrogen).

Clonogenic assays

One hundred thousand cells were seeded on 100 mm dishes with 10 ml media per dish (Li et al. 2004). On day 4, cells were treated with a PKCδ inhibitor or vehicle control for 6, 18, 24, or 48 h. Cells were trypsinized, counted via the trypan blue exclusion method in order to determine the number of live cells in the sample, and 500 live cells were seeded in triplicate onto 6-well plates. Cells were monitored for appropriate colony size and re-fed every 3–4 days.

We demonstrate in this report that human neuroendocrine tumor cell lines of pulmonary and gastrointestinal origin are sensitive to PKCδ inhibition. Knockdown of PKCδ by exposure to PKCδ-specific shRNA, or suppression of PKCδ activity by diverse small-molecule inhibitors, is sufficient to inhibit proliferation of these human neuroendocrine tumor cell lines and efficiently induce apoptosis.
At day 17, cells were stained with ethidium bromide (Guda et al. 2007) and counted using UVP LabWorks software (Upland, CA, USA).

**PKC kinase activity assays**

Assays were carried out using recombinant PKCα or PKCδ (Invitrogen) and the Omnia Kinase Assays (Invitrogen) with a ‘PKC kinase-specific’ peptide substrate. Incorporation of a chelation-enhanced fluorophore results in an increase in fluorescence (λex/360/λem/485) upon phosphorylation. The kit was used according to the manufacturer’s instructions.

**Reagents**

Rottlerin was purchased from EMD Biosciences (Darmstadt, Germany). The PKCδ inhibitor KAM1 is a chimeric molecule combining the chromene portion of rottlerin with the carbazole portion of staurosporine.

**Cell proliferation assays**

Cell proliferation was assessed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche). The number of viable cells growing in a single well on a 96-well microtiter plate was estimated by adding 10 μl MTT solution (5 mg/ml in PBS). After 4 h of incubation at 37 °C, the stain is diluted with 100 μl dimethyl sulfoxide. The optical densities are quantified at a test wavelength of 570 nm and a reference wavelength of 690 nm on a multiwell spectrophotometer. In some assays, MTS was used as substrate (Promega), and the absorbance of the product was monitored at 490 nm. Cell enumeration was carried out using a hemocytometer and viable cells were identified by trypan blue exclusion.

**Cytotoxicity assay**

Lactate dehydrogenase (LDH) release was assessed by spectrophotometrically measuring the oxidation of NADH in both the cells and the media. Cells were seeded in 24-well plates and exposed to PKC inhibitors or vehicle. After different times of exposure, cytotoxicity was quantified by a standard measurement of LDH release with the use of the LDH assay kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Briefly, total culture medium was cleared by centrifugation. For assay of released LDH, supernatants were collected. To assess total LDH in cells, Triton X-100 was added to vehicle (control) wells to release intracellular LDH. LDH assay reagent was added to lysates or supernatants and incubated for up to 30 min at room temperature in the dark, the reaction was stopped, and the absorbance was measured at 490 nm. The percentage of LDH release was then calculated as the LDH in the supernatants as a fraction of the total LDH.

**Immunoblot analyses**

Levels of proteins were measured and quantitated in carcinoid cell lines, as we have previously reported (Xia et al. 2007). Harvested cells were disrupted in a buffer containing 20 mM Tris (pH 7.4), 0.5% NP-40, and 250 mM NaCl. Total protein (40 μg) is separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes or PVDF membranes. Membranes are blocked overnight and probed with affinity-purified antibodies against PKC α and δ (BD Transduction Lab, Franklin Lakes, NJ, USA) or β-actin (Sigma). After washing, the blots were incubated with HRP-conjugated secondary antibodies and visualized using the Amersham enhanced chemiluminescence ECL system and quantitated by digital densitometry. Antibodies against human ERK, phospho-ERK, AKT, and phospho-Ser473-AKT were purchased from Cell Signaling (Danvers, MA, USA). GTP-bound Ras was assayed by affinity purification using a Raf1/RBD agarose conjugate (Upstate Biotechnology, Lake Placid, NY, USA) and detected with a pan-Ras antibody (Cell Signaling), following the manufacturer’s instructions.

**Downregulation of PKCδ by shRNA and lentiviral vectors**

shRNA knockdown of PKCδ and PKCα shRNA duplexes for PKCδ (shRNAs) are obtained from Qiagen. The shRNA sequences for targeting PKCδ are PKCδ-shRNA-1 (5'-GAUGAGAGGCCGUAGTTT-3') and PKCδ shRNA-2 (5'-GGCUGAGUUCCUG-CUGCAGTT-3') (Xia et al. 2007). The corresponding scrambled shRNAs were used as negative control. These shRNA sequences were also cloned into the pRNA6.1-Neo vector with a GFP tag according to the manufacturer’s instructions (GenScript, Piscataway, NJ, USA). shRNA for PKCα (PKC-PKCα-V6) are purchased from Upstate. Transfection of shRNA (oligo) is performed using 50 nM PKCδ shRNA, or the same amount of scrambled shRNA and Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Transfection of plasmid-based shRNA vectors was carried out using the same method. PKCδ protein levels were determined by immunoblot analysis (see below). The lentiviral vectors were previously described (Xia et al. 2009).
Statistical analysis

Experiments were carried out in triplicate for all experimental conditions. Data are shown as mean ± s.d. Where applicable, a two-tailed Student’s t-test or ANOVA was performed on the means of two sets of sample data and considered significant if P ≤ 0.05.

Results

PKCδ depletion by shRNA inhibits proliferation and induces cytotoxicity in human neuroendocrine cell lines

To determine the effects of specific PKCδ depletion on the proliferation and survival of human neuroendocrine tumor cell lines, PKCδ-specific shRNA was used to knock down PKCδ mRNA/protein. The cell lines studied for sensitivity included BON1, a human foregut (pancreatic) carcinoid tumor cell line; H727 cells, derived from a human bronchopulmonary carcinoid tumor; and the CNDT 2.5 cell line, a human cell line with neuroendocrine markers, initially described as a human midgut carcinoid tumor cell line. Exposure of BON1 and CNDT cell lines to PKCδ-specific shRNA in culture resulted in a profound inhibition of proliferation (Fig. 1A and B). In contrast, exposure of the same cells to a control (scrambled) shRNA did not affect proliferation. Efficient knockdown of PKCδ protein by specific shRNA was verified by immunoblotting.

To confirm and extend these experiments, lentiviral vectors containing the same shRNA sequences (PKCδ-specific or scrambled) were constructed. Infection of BON1, H727, and CNDT cell lines with these vectors demonstrated PKCδ-specific inhibition of proliferation (Fig. 2A–C). The lentiviral vector containing the scrambled sequence (control) consistently had a modest inhibitory effect on proliferation of both cell lines, but this never reached statistical significance. Efficient knockdown of PKCδ protein by the specific shRNA was verified by immunoblotting.

To determine whether the inhibition of tumor cell proliferation by PKCδ knockdown was accompanied by cytotoxic effects on the tumor cells, cytotoxicity in these cell lines was evaluated by quantitating LDH release. LDH, a stable cytoplasmic enzyme, is rapidly released into the cell culture medium after damage of the plasma membrane, and its level correlates quantitatively with the extent of cytotoxicity. Significant increases in LDH release/cytotoxicity were detected within 24 h of exposure to the lentiviral vector containing the PKCδ shRNA, and this release increased to approach the maximum possible LDH release (complete cell lysis, positive control) by 72 h (Fig. 3A–C). Only modest, but detectable, increases in LDH release were induced by the control (scrambled shRNA) lentiviral vector.

Figure 1 Effects of PKCδ knockdown by shRNA on proliferation of human neuroendocrine tumor BON1 and CNDT cells. BON1 (A) and CNDT 2.5 (B) cells were grown to 50% confluence in 96-well plates and then treated with PKCδ-shRNA or scrambled shRNA (sc-shRNA). The corresponding solvent equivalent volumes were used as vehicle controls (vehicle). After 48, 72, and 96 h of treatment, cell number was evaluated by MTS assay. (C) Immunoblot analysis of PKCδ protein levels in the same cell lines 72 h after exposure to lentivirus-transfected PKCδ-targeting shRNA or scrambled shRNA. Lentiviral PKCδ-targeted shRNA efficiently inhibited PKCδ protein expression.
Small-molecule inhibitors of PKCδ are cytotoxic to neuroendocrine tumor cell lines

We next determined whether a series of small-molecule PKCδ inhibitors would inhibit the growth of human neuroendocrine tumor cell lines. While not as specific for the PKCδ isozyme as technology employing genetic knockdown of the PKCδ mRNA and protein, such small-molecule inhibitors are more relevant for eventual therapeutic application.

Rottlerin is a naturally occurring product (Fig. 4A) that inhibits purified PKCδ at an IC$_{50}$ of 0.2–3.0 μM in vitro and inhibits PKCδ in cultured cells with an IC$_{50}$ of 5 μM in vivo (data not shown). It is relatively selective for PKCδ (PKCδ IC$_{50}$/PKCα IC$_{50}$ > 30:1; Gschwendt et al. 1994, Kikkawa et al. 2002), and this relative selectivity was confirmed in our in vitro assays (not shown). Furthermore, this compound not only directly inhibits purified PKCδ but also, over longer periods of exposure, significantly downregulates PKCδ protein specifically in cells, while having no effect on the levels of other PKC isozymes (Xia et al. 2007). Exposure to rottlerin produced a dose- and time-dependent decrease in cell number in the BON1, the CNDT 2.5, and the H727 cell lines, with an IC$_{50}$ of ~5 μM, by 48 h (not shown), and a significant reduction in relative cell numbers by 72 h (Fig. 4B). In contrast, rottlerin had no significant effect on the growth of two non-transformed human cell lines, MCF10 (breast epithelial cells) and PZ-HPV-7 (prostate epithelial cells; Fig. 4C). In addition, we have previously demonstrated that exposure to rottlerin under the same culture conditions has no significant effect on the growth of a number of other non-tumorigenic murine or human cells or cell lines (Xia et al. 2007).

Docking studies were conducted to predict how rottlerin binds to PKCδ. Rottlerin was docked into the catalytic binding site of several different PKC crystal structures. The structure of PKCδ complexed with staurosporine (pdb code 1XJD) was selected as the most suitable model. It is known from crystal structures of many kinase/inhibitor complexes that the kinase active site is flexible; therefore, regions known to be flexible were allowed to be free during the docking procedures. Chimeric molecules were designed using the PKCδ model developed from the rottlerin docking studies. The strategy was to retain most of the chromene part of rottlerin, which is assumed to give rottlerin its specificity but to vary the ‘head group’, which is assumed to bind to the hinge region of the kinase active site. A novel PKCδ inhibitor, KAM1, which is a chimeric molecule containing the substituted chromene portion of rottlerin and the N-alkylated carbazole portion of staurosporine (a non-selective pan-PKC inhibitor; Fig. 4A), was then tested for cytotoxic effects on neuroendocrine tumor cells. Comparative analyses of PKCδ-inhibitory activity demonstrated an in vitro IC$_{50}$ of 0.2 μM for rottlerin and an IC$_{50}$ of 0.9 μM for KAM1. In contrast, the PKCα IC$_{50}$ was >50 μM for each compound.
demonstrating some specificity for the novel isozyme PKCδ over classic isozyme PKCα. KAM1 produced a dose- and time-dependent decrease in cell number in the BON1, the CNDT 2.5, and the H727 cell lines, with an in vivo IC₅₀ of ~12 µM, by 48 h (not shown), and an 80% reduction in cell numbers by 72 h at the highest concentrations tested (Fig. 4A and B).

In parallel, cytotoxicity, as assessed by LDH release, was induced by exposure of the three carcinoid cell lines to rotterlin and to KAM1. In all three cell lines, cytotoxicity increased as a function of time and concentration of these inhibitors (Fig. 5A). As controls for the targeted nature of this approach, LDH release was assayed in NIH-3T3 cells (a non-transformed murine mesenchymal cell line) and in NIH-3T3 cells transformed by the introduction of an activated Ha-Ras allele (NIH-Ras). Consistent with previous reports, significant susceptibility to cytotoxicity after exposure to these PKCδ inhibitors was conferred in NIH cells by the presence of an activated Ras protein (Fig. 5B).

Ras signaling in neuroendocrine tumor cell lines

Because of their sensitivity to PKCδ inhibition and ‘Ras-mediated apoptosis,’ the activity of p21Ras protein in these neuroendocrine tumor cell lines was assessed by affinity pull-down of GTP-bound p21Ras species. Endogenous Ras activity (GTP-bound Ras) was high in the H727 cells and was not evident in the CNDT or BON1 cell lines, which contained GTP-bound p21Ras levels compared to those found in non-transformed cells (Fig. 6A).

It has been previously demonstrated that aberrant activation of certain Ras-signaling pathways, including the PI3K–AKT pathway and the Raf–MAPK pathway, is sufficient to render tumor cells susceptible to PKCδ inhibition, even in the absence of activating mutations of Ras itself (Xia et al. 2007). The activation status of downstream elements of these signaling pathways was, therefore, explored in these neuroendocrine tumor cell lines. Evidence for activation of Raf–MAPK, as defined by relative elevation of phospho-ERK levels, was observed in the H727 and CNDT lines (compared to the non-transformed negative control cell line MCF10; Fig. 6B). Evidence for some activation of PI3K signaling, as defined by activating phosphorylation of AKT (Ser473) relative to the non-transformed negative control cell line MCF10, was observed in all three neuroendocrine tumor cell lines.

Whether neuroendocrine tumor cell lines could escape from the anti-tumor actions of PKC inhibitors was explored by long-term exposure to the inhibitors in two experimental designs. In the first design, cells were plated at a lower density to allow monitoring over longer periods for potential growth. In these ‘continuous’ treatment studies, a PKCδ inhibitor was added at a ‘suboptimal’ concentration, and effects on proliferation were observed after exposure of 144 h (Fig. 7A and B). The decrease observed in the MTS signal from the control (vehicle treated) cells at 144 h represented both
overgrowth of these cultures and exhaustion of the culture media. In contrast, exposure of the human cell line BxPC3, which has wild-type Ras alleles, to the same PKCδ inhibitor did not affect its growth relative to vehicle alone (Fig. 7C). To allow evaluation over even longer periods of exposure, other cultures were re-fed with fresh growth medium containing the same PKCδ inhibitor at the same concentration. In these studies, growth-inhibitory effects persisted to 168 h of cumulative exposure (Fig. 7D and E).

The exposure time to PKCδ inhibition required for anti-tumor activity was assessed. BON1 and H727 cells were exposed to a suboptimal concentration of a PKCδ inhibitor for different intervals of time, the inhibitor was then washed out of the culture, and the effects on cell growth were assessed for the next 72 h. Differences in proliferation between rottlerin- and vehicle-treated cultures became statistically significant by 24 h of exposure and remained significant for all longer periods of exposure (Fig. 8A).

LDH release can be used to assess cytotoxic damage sufficient to compromise membrane integrity over a relatively short time span. An alternative method, which assesses lethal, but not necessarily immediate, cumulative damage to the tumor cell is a clonogenic assay. In this assay, tumor cells that remain viable after exposure to the compound are tested for their ability to proliferate sufficiently over time to form colonies of tumor cells. H727 cells were exposed to vehicle or a PKCδ inhibitor at suboptimal concentrations for varying durations. After re-plating of viable cells in media without inhibitor, colony numbers were quantitated over time. PKCδ inhibitors reduce the clonogenic capacity of H727 cells significantly after 6 h of exposure and the inhibitory effect remained significant for all subsequent exposure times (Fig. 8B). In parallel experiments, BON1 cells showed a similar drop-off in clonogenic capacity, reaching significance between 12 and 24 h of exposure to PKCδ inhibitors (not shown).
apoptosis. Specifically, aberrant signaling upstream pathways, will also sensitize cells to Ras-mediated activation of certain Ras downstream effector physiological activation of endogenous c-Ras, or Ras, we have more recently shown that supra-

Discussion

Ras mutations can be found in human malignancies with an overall frequency of 20%. A particularly high incidence of Ras gene mutations has been reported in malignant tumors of the pancreas (80–90%, K-Ras), in colorectal carcinomas (30–60%, K-Ras), in non-melanoma skin cancer (30–50%, H-Ras), and in hematopoietic neoplasias of myeloid origin (18–30%, K- and N-Ras; Serrano et al. 1997, Downward 2003). In the course of studying signaling by p21Ras, we discovered discrete anti-proliferative effects of p21Ras (Rake et al. 1991, Faller et al. 1994, Chen et al. 1996).

One of these properties is the activation of apoptotic signaling, resulting in rapid cell death, unless balanced by a simultaneous and independent activation of survival pathways (Chen & Faller 1995, 1996, 1999, Chen et al. 1998a,b, 2001, 2003, Liou et al. 2000, 2004, Ma et al. 2002). This Ras-generated apoptotic signaling specifically requires PKCδ activity (Xia et al. 2007, 2009). In contrast, PKCδ is not generally required for development or survival of normal tissues.

Although we first discovered these anti-proliferative activities of p21Ras as properties of activated oncogenic Ras, we have more recently shown that supra-physiological activation of endogenous c-Ras, or activation of certain Ras downstream effector pathways, will also sensitize cells to Ras-mediated apoptosis. Specifically, aberrant signaling upstream of Ras (e.g., via mutated growth factor receptors), or aberrant activation of Ras downstream pathways (PI3K or Raf–MEK), is sufficient to sensitize cells to apoptosis when PKCδ is suppressed (Xia et al. 2007, 2009).

Carcinoid and other neuroendocrine tumors of the bronchopulmonary/gastrointestinal tract share a number of the same genetic abnormalities (deletions and mutations) as adenocarcinomas (Arber et al. 1997, 1998).

Figure 5 Cytotoxic effects of PKCδ inhibitors on human neuroendocrine tumor cell lines. H727 cells (A) or NIH and NIH-Ras cells (B) were grown to 50% confluence in 96-well plates and then exposed to rottlerin or KAM1 at the concentrations indicated. After 24, 48, and 72 h of exposure, cell cytotoxicity was evaluated by LDH release assay (solid bars). Only the 72 h time point is shown for the NIH and NIH-Ras cells (panel B). Cells exposed to vehicle alone served as controls for determination of baseline LDH release values, which were subtracted at each time point. Total maximal LDH release was assigned the arbitrary value of 100%. Error bars represent S.E.M. P values for comparison between control (vehicle) and rottlerin or KAM1 effects on LDH release reached significance by 24 h of exposure (P ≤ 0.003) for the H727 and NIH-Ras cells and remained significant at the 48 and 72 h time points. In contrast, there were no statistically significant changes in LDH release for the NIH cells exposed to either compound, compared with control.

Figure 6 Ras signaling in neuroendocrine tumor cell lines. (A) p21Ras activity in neuroendocrine tumor cell lines. Nuclear-free lysates from NIH/3T3 cells (negative control), NIH/3T3-Ras cells (positive control), BON1 cells, H727 cells, and CNDT cells, containing a total of 400 μg protein from each indicated cell type, were used for analysis of Ras activity by Raf–RBD pull-down of GTP-bound p21Ras. Equal loading was demonstrated by re-probing the blot with anti-β-actin antibody. Pan-p21Ras protein expression levels were also analyzed. (B) Activation of Ras-signaling pathways in neuroendocrine tumor cell lines. Cell lysates from MCF10 cells (negative control), MCF10-Ras cells (positive control), BON1 cells, H727 cells, and CNDT cells were separated by SDS–PAGE, transferred to a membrane, and immunoblotted with antibodies against ERK, phospho-ERK, AKT, phospho-Thr308 AKT, and GAPDH (as a loading control).
Leotlela et al. 2003, Zikusoka et al. 2005). These abnormalities include activation of Ras directly by mutations, indirectly by loss of Ras-regulatory proteins such as NF-1, or via constitutive activation of growth factor receptors upstream of Ras or downstream effector pathways of Ras, such as PI3K and Raf/MAP kinase. Activation of (wild type) H-Ras and Ki-Ras is detected in a significant fraction of carcinoid and other gastrointestinal neuroendocrine tumors (65 and 10% respectively; Liedke et al. 1998, Maitra et al. 2000). Ras can be activated in neuroendocrine tumors by either point mutation (infrequently), constitutive signaling from upstream receptor tyrosine kinases, or loss of regulators of Ras, such as RasFLA or NF-1 (Stancu et al. 2003, Liu et al. 2005, Bausch et al. 2007). The Her-2/Neu tyrosine kinase receptor, which lies upstream of Ras, is amplified in up to 40% of gastric carcinoids and may identify more aggressive tumor types (Evers et al. 1992). The Raf/MAP kinase is found to be aberrantly activated in a fraction of neuroendocrine tumors. Activating mutations of B-Raf itself are found in some neuroendocrine tumors, but infrequently in carcinoid tumors (Perren et al. 2004, Tannapfel et al. 2005, Kunnimalaiyaan & Chen 2006, Karhoff et al. 2007). In those cases where activating point mutations of Raf are not present, however, activation of Raf and/or the Raf-substrate MAP kinases directly downstream of Raf, is common (Tannapfel et al. 2005, Karhoff et al. 2007). This activation of the Raf/MAP kinase pathway may have a causative role in the development of neuroendocrine tumors, independent of point mutations in B-Raf or Ras (Tannapfel et al. 2005). The PI3K pathway can be activated in neuroendocrine tumors by deletion of the tumor suppressor gene PTEN. Loss of PTEN in neuroendocrine tumors increases in frequency with the loss of differentiation in the tumor (Wang et al. 2005), and loss of PTEN expression may represent an important step in the progression of neuroendocrine tumors (Wang et al. 2002). Cyclin D1 upregulation in neuroendocrine tumors is quite common, likely as a result of Ras/Raf/MAP kinase pathway activation (Chung et al. 2000, Kawahara et al. 2002, Guo et al. 2003). Similarly, frequent coincident activation of the Ras effectors p38/MAP kinase and AKT/protein kinase B together have been reported (Chung et al. 2000, Kawahara et al. 2002, Guo et al. 2003).

Thus, as in many other human tumors, activation of Ras and Ras-signaling pathways likely contribute to tumor growth and progression in many neuroendocrine tumors. However, the activation of these pathways also makes these tumors dependent on Ras-related survival pathways, which require PKCδ for function. In the absence of this survival pathway, the proliferative...
properties of Ras signaling are re-directed toward apoptosis (Xia et al. 2007, 2009). We have shown in previous work that inhibition of PKC\(\delta\) protein or activity in non-transformed cells of multiple species by genetic knockdown, dominant-negative mutants, or small-molecule chemical inhibitors, does not affect their growth or clonogenic properties, suggesting that, by its selective toxicity toward aberrant Ras signaling, this approach is tumor targeted. Each of the three neuroendocrine tumor cell lines studied here had evidence for a different profile of Ras pathway activation, with elevated activity of p21Ras itself (as assessed by increases in GTP-bound p21Ras levels) and its downstream effector pathways in the H727 cells, activation of the Raf–MAPK pathway (as assessed by increases in phospho-ERK levels) in the CNDT cells, and some relative increases in PI3K signaling (as assessed by increases in phospho-AKT levels) in all three cell lines. Such heterogeneity in patterns of Ras pathway activation is common in most tumors, and each of these patterns of aberrant Ras signaling is sufficient to make tumor cells susceptible to apoptosis following PKC\(\delta\) downregulation (Xia et al. 2007).

In these studies, we have shown that neuroendocrine tumor cell lines are susceptible to growth inhibition and apoptosis when PKC\(\delta\) is downregulated by specific genetic modes (shRNA), or by less-specific, but potentially more clinically applicable, small-molecule inhibitors. Some of these small-molecule inhibitors have shown acceptable toxicity profiles in rodents (Fryer et al. 2002, Brown et al. 2005, Zhang et al. 2007; Douglas V Faller, Zhihong Chen & Yan Dai, unpublished observations). Washout studies suggest that duration of exposure to PKC\(\delta\) inhibitors of no more than 24 h is required to generate a significant effect on subsequent tumor cell proliferation. More importantly, significant reductions in tumor cell clonogenic capacity in two neuroendocrine cell lines were generated by exposure to a small-molecule inhibitor for as little as 6 h.

Rottlerin was identified as a protein kinase inhibitor that inhibited PKC\(\delta\) more potently than classic PKC isozymes, such as \(\alpha\) and \(\beta\) (Gschwendt et al. 1994, Keenan et al. 1997, Frasch et al. 2000). We have confirmed the greater inhibitory activity of rottlerin for PKC\(\delta\) relative to PKC\(\alpha\), using PKC proteins purified from mammalian cells, in previous work (Xia et al. 2007), as well as using recombinant PKC proteins in the current report. As inhibition of PKC\(\alpha\) is generally cytotoxic to all mammalian cells, their relative selectivity for PKC\(\delta\) may contribute to the lack of toxicity of rottlerin and related compounds on normal cells. To begin development of novel PKC\(\delta\) inhibitors,

Figure 8 Duration of exposure to PKC\(\delta\) inhibitors needed to inhibit tumor cell proliferation and clonogenic potential. (A) BON1 and H727 cells were grown to 30% confluence and then exposed to vehicle or to rottlerin (10 \(\mu\)M) for 6, 12, 24, 48, or 72 h. Media without inhibitor was replaced and cell numbers were estimated by MTS assay at 24, 48, and 72 h. Shown here are the results at 72 h of culture after each washout interval. Error bars represent S.E.M. Differences in proliferation between rottlerin- and vehicle-treated cultures became statistically significant \((P<0.01)\) by 24 h of exposure and remained significant for all longer periods of exposure. (B) Effects of PKC\(\delta\) inhibitor on tumor cell clonogenic capacity. H727 cells were grown to 30% confluence and then exposed to vehicle or rottlerin (10 \(\mu\)M) for 6, 12, 24, 48, or 72 h. Viable cells were enumerated and re-plated in media without inhibitor, and colony numbers were quantitated 96 h later. Error bars represent S.E.M. \(P\) values for comparison of DMSO control and rottlerin effects on clonogenic capacity reached significance \((P=0.0051)\) at 6 h of exposure and remained significant for all subsequent exposure times.
we carried out docking studies to predict how rottlerin binds to PKCδ. Rottlerin was docked into the catalytic binding site of several different PKC crystal structures. In many kinase/inhibitor complexes, the kinase active site is flexible; accordingly, regions known to be flexible were allowed to be free during the docking procedures. Chimeric molecules were designed using the PKCδ model developed from the rottlerin docking studies. The strategy was to retain most of the ‘bottom’ part of rottlerin, which was assumed to give rottlerin its specificity, but to vary the ‘head group,’ which was assumed to bind to the hinge region of the kinase active site. A novel PKCδ inhibitor, KAM1, which is a chimeric molecule possessing portions of rottlerin and staurosporine (a non-selective pan-PKC inhibitor), was synthesized. This novel chimeric molecule demonstrated some PKCδ/PKCζ-inhibitory selectivity and accordingly produced cytotoxic effects on neuroendocrine tumor cells. SAR studies of this molecule are ongoing, with the goal of developing even more selective and potent PKCδ inhibitors as potential therapeutics for carcinoid tumors.

Gastrointestinal and pulmonary carcinoid tumors are uncommon, but unfortunately are generally refractory to conventional cytotoxic chemotherapeutic and radiotherapeutic approaches. A targeted therapeutic approach, such as induction of Ras-mediated apoptosis by PKCδ inhibition, which selectively takes advantage of the very oncogenic mutations, which contribute to the malignancy of the tumor, may have potential as a novel and selective therapeutic modality for these malignancies.

Declaration of interest

D V Faller is an inventor on patent applications relating to this work. The authors declare that they have no other competing interests.

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Author contribution statement

D V Faller, Z Chen, and L W Forman designed the studies and evaluated the results. Z Chen, L W Forman, and A Takashima performed the experiments; R A Bohacek performed molecular modeling. K A Miller, B English and R M Williams designed and synthesized compounds. D V Faller, Z Chen, and L W Forman wrote the manuscript. All authors read and approved the final manuscript.

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