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

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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 p21^{Ras}-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 signaling sensitizes cells to apoptosis when the activity of the PKC δ isozyme is suppressed and that PKC δ suppression is not toxic to cells with normal levels of p21^{Ras} signaling. We demonstrate here that inhibition of PKC δ by a number of independent means, including genetic mechanisms (shRNA) or small-molecule inhibitors, is able to efficiently and selectively repress the growth of human neuroendocrine cell lines derived from bronchopulmonary, foregut, or hindgut tumors. PKC δ inhibition in these tumors also efficiently induced apoptosis. Exposure to small-molecule inhibitors of PKC δ over a period of 24 h is sufficient to significantly suppress cell growth and clonogenic capacity of these tumor cell lines. Neuroendocrine tumors are typically refractory to conventional therapeutic approaches. This Ras-targeted therapeutic approach, mediated through PKC δ suppression, which selectively takes advantage of the very oncogenic mutations that contribute to the malignancy of the tumor, may hold potential as a novel therapeutic modality.

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Introduction

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. At least 30% of all human malignancies display activating mutations in the *RAS* genes, and perhaps another 60% display other activating mutations in, or over-activity of, p21^{Ras}-signaling pathways. We previously reported

that aberrant activation of Ras results in an absolute dependency on PKC δ -mediated survival pathways (Xia *et al.* 2007, 2009). Over-activity of Ras signaling, therefore, sensitizes tumor cells to apoptosis induced by suppression of PKC δ activity, whereas suppression of PKC δ 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 $\sim$ 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 PKC δ inhibition. Knockdown of PKC δ by exposure to PKC δ -specific shRNA, or suppression of PKC δ 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 L-glutamine (Invitrogen, Carlsbad, CA, USA), 200 U penicillin/ml; 200 μ g streptomycin/ml (Invitrogen); 10 ng/ml nerve growth factor (Invitrogen); 1 $\times$ MEM non-essential amino acids (Cellgro); 1 $\times$ 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 μ g 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 PKC δ 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.

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 PKC α or PKC δ (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 ($\lambda_{\text{ex}}360/\lambda_{\text{em}}485$) upon phosphorylation. The kit was used according to the manufacturer’s instructions.

Reagents

Rottlerin was purchased from EMD Biosciences (Darmstadt, Germany). The PKC δ 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 μ l MTT solution (5 mg/ml in PBS). After 4 h of incubation at 37 °C, the stain is diluted with 100 μ l 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

Lactose 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 δ 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 μ g) 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 PKC δ by shRNA and lentiviral vectors

shRNA knockdown of PKC δ and PKC α shRNA duplexes for PKC δ (shRNAs) are obtained from Qiagen. The shRNA sequences for targeting PKC δ are PKC δ -shRNA-1 (5'-GAUGAAGGAGGCGCUCAGTT-3') and PKC δ shRNA-2 (5'-GGCUGAGUUCUGG-CUGGACTT-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 PKC α (PKC-PKC α -V6) are purchased from Upstate. Transfection of shRNA (oligo) is performed using 50 nM PKC δ 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. PKC δ 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 $\pm$ 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 $P \leq 0.05$.

Results

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

To determine the effects of specific PKC δ depletion on the proliferation and survival of human neuroendocrine tumor cell lines, PKC δ -specific shRNA was used to knock down PKC δ 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 PKC δ -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 knock-down of PKC δ protein by specific shRNA was verified by immunoblotting.

To confirm and extend these experiments, lentiviral vectors containing the same shRNA sequences (PKC δ -specific or scrambled) were constructed. Infection of BON1, H727, and CNDT cell lines with these vectors demonstrated PKC δ -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 PKC δ protein by the specific shRNA was verified by immunoblotting.

To determine whether the inhibition of tumor cell proliferation by PKC δ 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 PKC δ 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 PKC δ 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 PKC δ -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 PKC δ protein levels in the same cell lines 72 h after exposure to lentivirus-transfected PKC δ -targeting shRNA or scrambled shRNA. Lentiviral PKC δ -targeted shRNA efficiently inhibited PKC δ protein expression.

Figure 2 Effects of PKC δ knockdown by PKC δ -specific shRNA lentivirus on proliferation of human neuroendocrine tumor cells. BON1 (A), CNDT 2.5 (B), and H727 (C) cells were grown to 50% confluence in 96-well plates and then infected with PKC δ -shRNA-lentivirus (PKC δ shRNA) or scrambled shRNA lentivirus (sc-shRNA). Cells exposed to mock lentiviral infection (vehicle) also served as controls. After 24, 48, and 72 h of treatment, cell proliferation was evaluated by MTS assay. Control values were normalized to 100%. Error bars represent s.e.m. *P* values for comparison between control (scrambled shRNA) lentivirus and PKC δ -shRNA lentivirus effects on cell number reached significance at 24 h of exposure ($P \leq 0.001$) for all cell lines and remained significant at the 48 and 72 h time points.

Small-molecule inhibitors of PKC δ are cytotoxic to neuroendocrine tumor cell lines

We next determined whether a series of small-molecule PKC δ inhibitors would inhibit the growth of human neuroendocrine tumor cell lines. While not as specific for the PKC δ isozyme as technology employing genetic knockdown of the PKC δ 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 PKC δ at an IC_{50} of 0.2–3.0 μ M *in vitro* and inhibits PKC δ in cultured cells with an IC_{50} of 5 μ M *in vivo* (data not shown). It is relatively selective for PKC δ (PKC δ IC_{50} /PKC α 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 PKC δ but also, over longer periods of exposure, significantly downregulates PKC δ 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 $\sim$ 5 μ M, 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 PKC δ . Rottlerin was docked into the catalytic binding site of several different PKC crystal structures. The structure of PKC θ 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 PKC δ 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 PKC δ 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 PKC δ -inhibitory activity demonstrated an *in vitro* IC_{50} of 0.2 μ M for rottlerin and an IC_{50} of 0.9 μ M for KAM1. In contrast, the PKC α IC_{50} was > 50 μ M for each compound,

Figure 3 Cytotoxic effects of PKC δ knockdown by shRNA-lentivirus on human neuroendocrine tumor cell lines. BON1 (A), CNDT 2.5 (B), and H727 (C) cells were grown to 50% confluence in 96-well plates and then infected with PKC δ -shRNA-lentivirus or scrambled shRNA lentivirus (vector). Cells exposed to mock lentiviral infection (vehicle) also served as controls. After 24, 48, and 72 h of treatment, cell cytotoxicity was evaluated by LDH release assay. Total maximal LDH release was assigned the arbitrary value of 100%. Error bars represent s.e.m. *P* values for comparison between control (scrambled shRNA) lentivirus and PKC δ -shRNA lentivirus effects on LDH release reached significance at 24 h of exposure ($P \leq 0.004$) for all cell lines and remained significant at the 48 and 72 h time points.

demonstrating some specificity for the novel isozyme PKC δ over classic isozyme PKC α . 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 μ M, 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 rottlerin 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 PKC δ 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 PKC δ inhibition and ‘Ras-mediated apoptosis,’ the activity of p21^{Ras} protein in these neuroendocrine tumor cell lines was assessed by affinity pull-down of GTP-bound p21^{Ras} 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 p21^{Ras} 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 PKC δ 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 PKC δ 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

Figure 4 Effects of PKC δ inhibitors on proliferation of human neuroendocrine tumor cell lines. (A) Structures of rottlerin and KAM1, a rottlerin/staurosporine chimera. Neuroendocrine tumor cell lines (B) or non-transformed human cell lines MCF10 or PZ-HPV-7 (PZ-7) (C) were grown to 80% confluence in 96-well plates and then exposed to rottlerin or KAM1 at 5, 10, 20, or 40 μ M (open bars). The corresponding equivalent volumes of solvent (DMSO) were used as vehicle controls (Con; solid bars). After 72 h of exposure, cell growth was evaluated by MTT assay or enumeration of viable cells. Control values were normalized to 100%. *P* values for comparison between control vehicle and rottlerin effects on cell number in the neuroendocrine tumor cell lines reached significance at 24 h of exposure ($P \leq 0.004$) for all cell lines and remained significant at the 48 and 72 h time points. *P* values for comparison between control vehicle and KAM1 effects on cell number in the neuroendocrine tumor cell lines reached significance by 72 h of exposure ($P \leq 0.02$) for all cell lines. In contrast, in the non-transformed human cell lines, neither exposure to rottlerin or KAM1 produced a statistically significant effect on cell proliferation. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-10-0224>.

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 PKC δ 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 PKC δ 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 PKC δ inhibition required for anti-tumor activity was assessed. BON1 and H727 cells were exposed to a suboptimal concentration of a PKC δ 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 PKC δ inhibitor at suboptimal concentrations for varying durations. After re-plating of viable cells in media without inhibitor, colony numbers were quantitated over time. PKC δ 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 PKC δ inhibitors (not shown).

Figure 5 Cytotoxic effects of PKC δ 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 ($P \leq 0.003$) 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.

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 p21^{Ras}, we discovered discrete anti-proliferative effects of p21^{Ras} (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 PKC δ activity (Xia *et al.* 2007, 2009). In contrast, PKC δ is not generally required for development or survival of normal tissues.

Although we first discovered these anti-proliferative activities of p21^{Ras} 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 PKC δ 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,

Figure 6 Ras signaling in neuroendocrine tumor cell lines. (A) p21^{Ras} 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 p21^{Ras}. Equal loading was demonstrated by re-probing the blot with anti-β-actin antibody. Pan-p21^{Ras} 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).

Figure 7 Effects of longer term exposure of human neuroendocrine tumor cell lines to PKC δ inhibitors. BON1 cells (A and D) or CNDT cells (B and E) or BxPC3 cells (C) were exposed to rottlerin at a suboptimal concentration (10 μ M). Cells exposed to vehicle (DMSO) alone served as controls. At the indicated time points, cell numbers were estimated by MTS assays or enumeration of viable cells. In the cultures depicted in panels A, B, and C, media was not changed. In the cultures depicted in panels D and E, fresh media containing the PKC δ inhibitor was replaced after 72 h of exposure (arrows). Error bars represent S.E.M. (Fall off in cell numbers in control cultures at the longest time points likely reflect overgrowth observed in the control cultures.).

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 RASSF1A 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 PKC δ for function. In the absence of this survival pathway, the proliferative

Figure 8 Duration of exposure to PKC δ 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 μ 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 δ inhibitor on tumor cell clonogenic capacity. H727 cells were grown to 30% confluence and then exposed to vehicle or rottlerin (10 μ 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.

properties of Ras signaling are re-directed toward apoptosis (Xia *et al.* 2007, 2009). We have shown in previous work that inhibition of PKC δ 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 p21^{Ras} itself (as assessed by increases in GTP-bound p21^{Ras} 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 δ 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 δ 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 δ 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 δ more potently than classic PKC isozymes, such as α and β (Gschwendt *et al.* 1994, Keenan *et al.* 1997, Frasch *et al.* 2000). We have confirmed the greater inhibitory activity of rottlerin for PKC δ relative to PKC α , 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 α is generally cytotoxic to all mammalian cells, their relative selectivity for PKC δ may contribute to the lack of toxicity of rottlerin and related compounds on normal cells. To begin development of novel PKC δ inhibitors,

we carried out docking studies to predict how rottlerin binds to PKC δ . 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 PKC δ 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 PKC δ 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 PKC δ /PKC α -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 PKC δ 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 PKC δ 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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