Effects of protein kinase C modulation by PEP005, a novel ingenol angelate, on mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling in cancer cells

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Abstract

PEP005 (ingenol-3-angelate) is a novel anticancer agent extracted from *Euphorbia peplus* that was previously shown to modulate protein kinase C (PKC), resulting in antiproliferative and proapoptotic effects in several human cancer cell lines. In Colo205 colon cancer cells, exposure to PEP005 induced a time- and concentration-dependent decrease of cells in S phase of cell cycle and apoptosis. In Colo205 cells exposed to PEP005, a variety of signaling pathways were activated as shown by increased phosphorylation of PKCδ, Raf1, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (MAPK), c-Jun NH_2-terminal kinase, p38 MAPK, and PTEN. PEP005-induced activation of PKCδ was associated with its translocation from the cytosol to the nucleus and other cellular membranes. Interestingly, PEP005 treatment also resulted in reduced expression of PKCα and reduced levels of phosphorylated active form of AKT/protein kinase B. These data suggest that PEP005-induced activation of PKCδ and reduced expression of PKCα resulted in apoptosis by mechanisms mediated by activation of Ras/Raf/MAPK and inhibition of the phosphatidylinositol 3-kinase/AKT signaling pathways. This study supports ongoing efforts targeting PKC isoforms in cancer therapy with PEP005 alone and in combination with other cytotoxic agents. [Mol Cancer Ther 2008;7(4):915–22]

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Introduction

The protein kinase C (PKC) family of isoenzymes is a serine-threonine kinase that participates in multiple cell signaling pathways involved in the control of many cellular processes, including proliferation, differentiation, senescence, invasion, and apoptosis, in both normal and cancer cells (reviewed in refs. 1, 2). The PKC enzyme family comprises >11 isoforms that have been divided into three groups based on their interactions with calcium and diacylglycerol as cofactors. Classic PKCs, including isoforms α, βI, βII, and γ, require both calcium and diacylglycerol for activation. Novel PKCs, including isoforms δ, ε, η, and θ, are independent of calcium but require diacylglycerol for activation. Atypical PKCs, including λ and ζ, are calcium and diacylglycerol independent. There is now substantial evidence that the different PKC isoforms play specific roles in cell regulation with, for example, PKCδ involved in regulation of cell survival (3–5). PKCθ is activated during T lymphocyte activation (6) and PKCα and PKCβII are both being implicated in the regulation of cell proliferation (7–9). Once activated, PKC can transmit signals to the nucleus via cross-talk with the mitogen-activated protein kinase (MAPK) signaling pathway, which may incorporate Raf1, MAPK/extracellular signal-regulated kinase (ERK) kinases (MEK), ERKs, c-Jun NH_2-terminal kinase (JNK), and p38 MAPK (10). ERK activation can in turn stimulate transcription factors, such as myc, myb, max, fos, and jun (11), enabling the expression of genes encoding for enzymes required for key metabolic functions (12). Activation of JNK and p38 MAPK by PKC can activate proliferation, differentiation, and apoptosis in cells (13, 14). Thus, PKCs are known to play an important role in modulating proliferation, invasion, and apoptosis in several cellular models either directly or via interaction with other signaling pathways.

Apoptosis is modulated by PKC isoforms through a balance between proapoptotic and antiapoptotic signals. Both activation and inhibition of PKCs have been associated with apoptosis (15). PKCα and PKCβII seem to be antiapoptotic in most cells. PKCα is inactivated by proapoptotic factors, including ceramide (7), and is known to phosphorylate bcl-2, potentiating its antiapoptotic function in mitochondria (8). In contrast, PKCδ has a proapoptotic function as it is activated proteolytically by

caspase-3 and is involved in the nuclear phase of apoptosis (4, 16). Contrary to its well-documented role as a pro-apoptotic mediator, PKCδ may also, in some circumstances, exert antiapoptotic functions. For example, PKCδ was shown to participate in the tumor necrosis factor-α antiapoptotic signaling in human neutrophils and in the fibroblast growth factor antiapoptotic signaling in PC12 prostate cancer cells, and it was associated with the outgoing of apoptosis of B-cell chronic lymphocytic leukemia (3). Considering the importance of PKCs in apoptosis induction, a thorough understanding of how PKCδ and PKCα isoforms regulate apoptosis should help in the identification of novel and more efficient anticancer therapy.

Although targeted therapy has shown potent antitumor activity in a variety of solid tumors, including breast, colon, and lung cancer, resistance to those agents requires the appraisal of novel molecular targeted therapeutics, including drugs that interfere with signal transduction for cell survival or apoptosis such as the PKC signaling pathway (17). PEP005 is an ingenol angelate extracted and purified from *Euphorbia peplus*, a plant used in traditional medicine (18, 19). PEP005 is being developed as a topical treatment for actinic keratoses and basal cell skin carcinoma in patients and as a systemic treatment for leukemia in preclinical models (20–22). At relatively high concentrations, PEP005 induces mitochondrial disruption and cell death (20, 23). PEP005 is a potent activator of classic and novel PKC isoenzymes (24), which shows effects in whole cells that are distinct from the phorbol ester phorbol 12-myristate 13-acetate (PMA). PEP005 induces biphasic pattern of induction of interleukin-6 compared with the typical phorbol ester PMA. Moreover, PEP005 induces a distinct pattern of PKC translocation compared with PMA, producing a rapid nuclear translocation of PKCδ, whereas PMA induces initial translocation to the cell membrane (24). PEP005 induced apoptosis in acute myeloid leukemia cell lines, whereas PMA induced leukemic cell differentiation (21).

Compared with data accumulated in hematologic malignancies, less is known about PEP005 activity in solid tumor models: previous studies have shown that high concentrations (micromolar) of PEP005 induce cell death in human breast and prostate cancer cells (20). The aim of this study was to characterize the effects of PEP005 in a panel of human solid tumor cell lines and to evaluate PEP005 effects on intracellular signaling in sensitive and resistant cancer cell lines.

Materials and Methods

Materials

PEP005 (ingenol-3-angelate) was supplied by Peplin Ltd. PMA, rottlerin, wortmannin, UO126, chelerythrine chloride, and okadaic acid were purchased from Sigma. SB203580 and SP600125 were obtained from Calbiochem.

Cell Lines

HT29, MCF7, and OVCAR3 cell lines were obtained from the American Type Culture Collection. HCT116, Colo205, HCC2998, HOP62, HOP92, IGROV1, and MDA-MB-435 cell lines were obtained from the National Cancer Institute collection. H1299 human lung cancer cell line was kindly provided by R. Fahraeus (U716, Paris, France). Cells were grown as monolayers in RPMI 1640 supplemented with 10% FCS (Invitrogen), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere and regularly checked for the absence of *Mycoplasma*.

In vitro Growth Inhibition Assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay]

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out as described previously (25). In brief, cells were seeded in 96-well tissue culture plates at a density of 2×10^3 per well. Cell viability was determined after 120 h of incubation by the colorimetric conversion of yellow, water-soluble tetrazolium MTT (Sigma) into purple, water-insoluble formazan. This reaction is catalyzed by mitochondrial dehydrogenases and is used to estimate the relative number of viable cells (26). Cells were incubated with 0.4 mg/mL MTT for 4 h at 37°C. After incubation, the supernatant was discarded, the cell pellet was resuspended in 0.1 mL DMSO, and the absorbance was measured at 560 nm using a microplate reader (Dynatech). Wells with untreated cells or with drug-containing medium without cells were used as positive and negative controls, respectively. Growth inhibition curves were plotted as a percentage of untreated control cells.

Cell Cycle Analysis and Apoptosis Assays

Cell cycle analysis and the measurement of the percentage of apoptotic cells were assessed by flow cytometry. In brief, cells were seeded onto 25 cm^3 flasks and treated with various concentrations of PEP005. At various time points, adherent and nonadherent cells were recovered, washed with PBS, fixed in 70% ethanol, and stored at +4°C until use. Cells were rehydrated in PBS and incubated for 20 min at room temperature with 250 μg/mL RNase A and for 20 min at +4°C with 50 μg/mL propidium iodide in the dark. The cell cycle distribution and percentage of apoptotic cells were determined with a flow cytometer [FACS-Calibur and CellQuest Pro software (Becton Dickinson)]. Apoptosis was confirmed using the Annexin V-FITC Apoptosis Detection kit (Sigma).

Western Blot Analysis

Cells were lysed in buffer containing 50 mmol/L HEPES (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L vanadate, 100 mmol/L NaF, and 0.40 mg/mL phenylmethylsulfonyl fluoride. Equal amounts of protein (20 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk or 5% bovine serum albumin in 0.05% Tween 20/TBS and then incubated with the first antibody overnight. Membranes were then washed thrice with 0.05% Tween 20 in TBS and incubated with the secondary antibody conjugated to anti-mouse or anti-rabbit horseradish peroxidase. Bands were visualized by using the enhanced chemiluminescence Western blotting detection system (Amersham). Band intensities were quantified

Table 1. Inhibitory effects of PEP005 in a panel of characterized cell lines

Cell line	Main characteristics*			PEP005 IC_{50} ± SD (μmol/L)		
	p53	PKCα	PKCδ	1 h	24 h	48 h
Colon						
HT29	mut	Low	High	240 ± 28	110 ± 35	140 ± 28
HCT116	wt	Low	Low	>400	>400	120 ± 24
Colo205	mut	High	Int	20 ± 7	23 ± 8	0.01 ± 0.002
HCC2998	mut	Low	High	31 ± 9	40 ± 14	30 ± 6
Breast						
MCF7	wt	Low	Low	290 ± 60	210 ± 36	180 ± 36
MDA-MB-435	mut	Low	High	90 ± 23	95 ± 30	2.6 ± 0.52
Ovarian						
OVCAR3	mut	High	Low	350 ± 85	270 ± 50	200 ± 40
IGROV1	wt	Low	Low	250 ± 65	270 ± 62	200 ± 42
Lung						
Hop62	mut	High	Low	200 ± 46	110 ± 35	110 ± 22
Hop92	mut	High	High	230 ± 78	210 ± 52	85 ± 17

Abbreviations: wt, wild-type; mut, mutated; Int, intermediate expression in relation to average for 60 cancer cell lines.
*National Cancer Institute screen.

using a Scion Image for Windows (Scion Corp.)[7] and normalized relative to actin. Densitometric analysis was done under conditions that yielded a linear response.

The following first antibodies were used: anti-PKCs (α, β, γ, δ, ε, and η), anti-phospho-PKCδ, anti-cleaved caspase-3, anti-PTEN, anti-phospho-PTEN, anti-phospho-MAPK, anti-AKT, anti-phospho-AKT, and anti-phospho-Raf1 (all purchased from Cell Signaling); anti-phospho-JNK (Santa Cruz Biotechnology); anti-phospho-p38 MAPK (BD Biosciences); and anti-phospho-PKCα (Upstate Biotechnology). All antibodies were used at a 1:1,000 dilution except for anti-PKCβ, which was used at a 1:250 dilution.

Cellular Fractionation

Cells were incubated for 30 min with 20 nmol/L PMA or 0.3 μmol/L PEP005, washed twice in PBS, and then lysed by homogenization in hypotonic lysis buffer in the absence of detergent. Nuclei were isolated by centrifugation at 1,000 × *g* for 10 min. Centrifugation at 100,000 × *g* for 45 min at 4°C was used to isolate the cytosol (supernatant) and cell membrane (pellet) fractions. All were taken up in SDS sample buffer and analyzed for PKCδ by Western blotting.

Reverse Transcription-PCR

Real-time quantitative reverse transcription-PCR was done using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems). Briefly, total RNA was reverse transcribed before real-time PCR amplification. The transcripts of the gene coding for the TATA box-binding protein were used as the endogenous RNA control, and each sample was normalized based on its TATA box-binding protein content. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min. Experiments were done in duplicate.

[7] http://www.scioncorp.com

Results

PEP005-Induced Inhibitory Effects, Cell Cycle Changes, and Apoptosis in Human Cancer Cells

The inhibitory activity of PEP005 was determined by MTT assay in a panel of 10 cancer cell lines exposed at concentrations ranging from 0.001 to 300 μmol/L for 1, 24, and 48 h (Table 1). Three of the cell lines, Colo205, HCC2998, and MDA-MB-435, were very sensitive to PEP005, with antiproliferative effects achieved with prolonged exposure to PEP005 (≥24 h) and IC_{50} values in the range of 0.01 to 30 μmol/L. The remaining seven cell lines were relatively insensitive to PEP005 with IC_{50} values in excess of 100 μmol/L (Table 1). Effects of PEP005 on cell cycle were studied further in Colo205 cells that were incubated with 0.03 and 0.3 μmol/L of PEP005 for 1, 5, 12, 24, and 48 h. PEP005 exposure led to a time-dependent decrease of cells in S phase observed after 12 h (Fig. 1A). In this cell line, apoptosis induction was investigated after 24, 48, and 72 h of exposure by Annexin V/propidium iodide staining (Fig. 1B). Twenty-four hours of exposure to PEP005 resulted in apoptosis followed by eventual induction of cellular necrosis after ≥48 h of exposure.

The caspase family is a postaspartate-cleaving cysteine protease, which is required for apoptosis in several experimental systems (27). Recently, PKCδ was shown to be a substrate for caspase-3, and caspase-3 cleavage was described as being associated with PKCδ-induced apoptosis (28). To study the role of caspase-3 in PEP005-induced apoptosis, Colo205 cells were incubated for 20, 30, 40, and 60 min and 24 h with 0.3 μmol/L PEP005. The cleaved fragment of activated caspase-3 was detected after 40 min of incubation with PEP005 (Fig. 1C).

It was recently shown that p53-dependent DNA damage-induced apoptosis was regulated by PKCδ (29). To further determine whether p53 played a role in PEP005-induced apoptosis, we transfected p53-null human H1299 lung

carcinoma cells with plasmid containing p53 but observed no significant modification in PEP005-induced cytotoxicity (Fig. 1D).

Our results show that PEP005 induces cell cycle arrest and apoptosis in Colo205 colon cancer cell line via a mechanism that seems p53 independent. Further study of molecular mechanisms leading to cell death in response to PEP005 requires the identification of target and downstream signaling pathways.

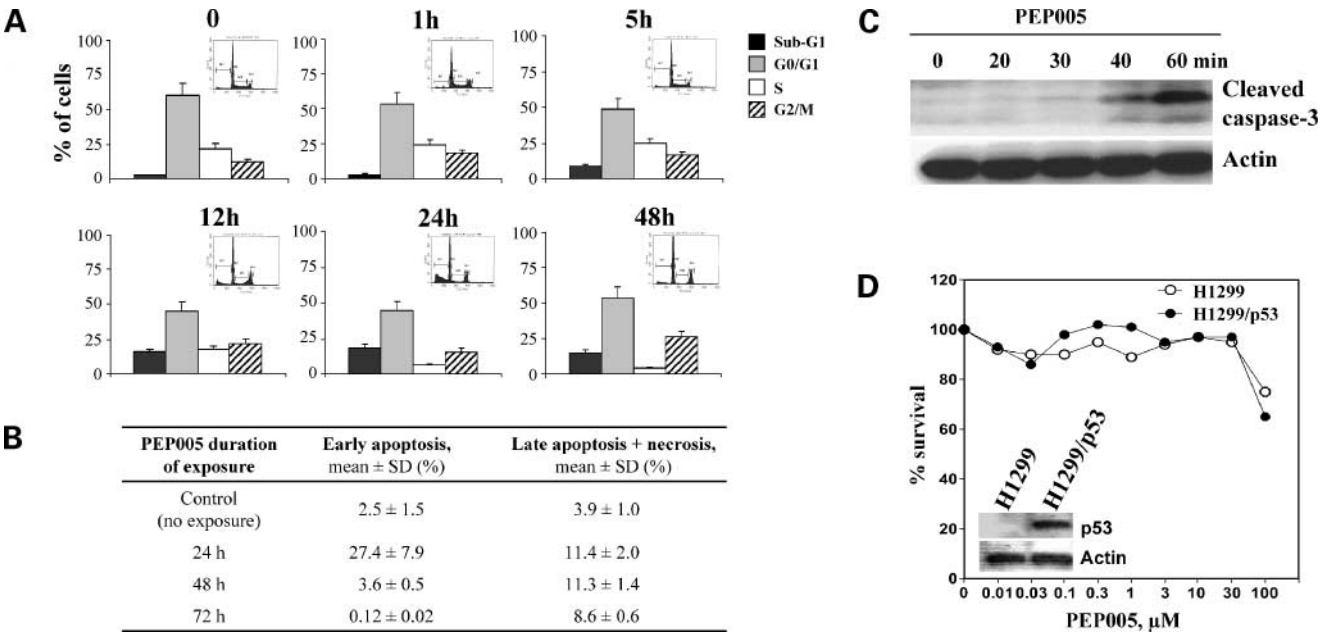
Role of PKC Isoforms in Sensitivity to PEP005

PKC Isoform Profiling in Solid Tumor Cell Lines. Previous studies have established that PEP005 is a modulator of PKC signaling pathways (24). We therefore determined if the antiproliferative effects of PEP005 in 10 solid tumor cell lines were related to differential expression of PKC isoenzymes. As shown in Fig. 2A, the panel of tumor cell lines expressed significant levels of PKCα, PKCβ, PKCγ, and PKCδ. PKCε was only expressed at a low level and PKCη was only detected at low level in three cell lines (HCT116, Colo205, and HCC2998). The cell lines did not display major differences in expression of PKCβ, PKCγ, or PKCε expression. In contrast, Colo205 cells showed the highest expression of PKCα, which was also observed at a high level in HOP62 and HOP92 cells. The highest levels of PKCδ expression were observed in Colo205 and MDA-MB-435 cell lines.

Effects of PEP005 on PKC Isoforms in Colo205 Cells. Prolonged treatment with phorbol esters and other natural products is known to result in down-regulation of several PKC isoforms (30). We determined whether there was any isoform that may be selectively down-regulated by treatment with PEP005 in Colo205 cells and compared this with the classic PKC agonist PMA. Because PMA is much more potent PKC modulator than PEP005, this study used high concentrations of PEP005 to obtain comparable biological effects. The PEP005-sensitive cell line Colo205 was treated with PMA (100 nmol/L) or PEP005 (3 μmol/L) for 4 h and then postincubated in R10 drug-free medium for 24 h. Protein levels of PKCα and PKCδ were only slightly decreased by PMA and were strongly down-regulated by PEP005 (Fig. 2B). As shown in Fig. 2C, PEP005 also down-regulated the active phosphorylated form of PKCα and PKCδ after long-term culture (>5 h) following the initial increase in phosphorylated PKCs at 20 and 30 min of treatment. The PKCα down-regulation was carried out at posttranscriptional level because quantitative reverse transcription-PCR did not show any changes in PKCα gene expression after PEP005 treatment (data not shown). The activation of PKCδ by PEP005 was followed by its translocation from the cytosol to nucleus and membranes. PEP005 caused rapid (30 min) translocation from the cytoplasm to the nuclear membrane. This was followed by the appearance of PKCδ in the plasma membrane (Fig. 2D). Compared with PEP005, PMA treatment caused similar effects on translocation of PKCδ.

PEP005 Downstream Intracellular Signaling

In the next series of experiments, we investigated the possible downstream signaling events from PKC, exploring



B

PEP005 duration of exposure	Early apoptosis, mean ± SD (%)	Late apoptosis + necrosis, mean ± SD (%)
Control (no exposure)	2.5 ± 1.5	3.9 ± 1.0
24 h	27.4 ± 7.9	11.4 ± 2.0
48 h	3.6 ± 0.5	11.3 ± 1.4
72 h	0.12 ± 0.02	8.6 ± 0.6

Figure 1. Cell cycle changes and mechanisms of cell death induced by PEP005 in Colo205 cells. **A,** Colo205 cells were treated with 0.3 μmol/L PEP005 for 1, 5, 12, 24, and 48 h, fixed, and stained with propidium iodide. Cell cycle effects were analyzed by flow cytometry. **B,** Colo205 cells were incubated with 1 μmol/L PEP005 for the durations shown and apoptotic and necrotic cells were assessed by binding of Annexin V-FITC and uptake of propidium iodide as described in Materials and Methods. **C,** PEP005 induces caspase-3 activation in Colo205 cells. Cellular extracts were prepared from Colo205 cells treated with 0.3 μmol/L PEP005 for 20, 30, 40, and 60 min. Analysis by Western blotting of caspase-3 activation revealed 17-kDa cleaved fragments. The blot shown is representative of three separate experiments done. **D,** Western blot of p53 in H1299 and H1299/p53 cell extracts. The cytotoxicity of PEP005 was evaluated after 48 h of treatment.

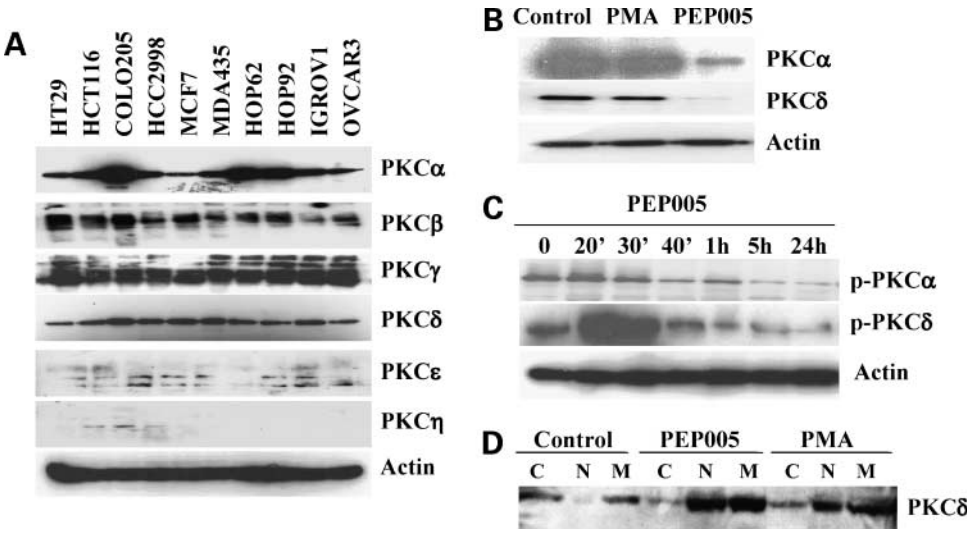


Figure 2. Effects of PEP005 on the PKC signaling pathway. **A,** basal protein expression of different PKC isoforms in 10 solid tumor cell lines. **B** to **D,** effects of PEP005 exposure on expression and activation of PKCs. **B,** Colo205 cells were treated with PMA (20 nmol/L) or PEP005 (3 μmol/L) for 4 h, and then protein extracts were then tested for PKCα, PKCδ, PKCε, and actin expression. **C,** Colo205 cells exposed to 0.3 μmol/L PEP005 for the indicated time were analyzed by Western blot for activation of PKCα and PKCδ. **D,** PEP005 induced activation of PKCδ by translocation from the cytosol (*C*) to the nucleus (*N*) and other cellular membranes (*M*). The blots shown are representative of three separate experiments done.

the changes of phosphorylation levels of member of the MAPK signaling pathways (Raf, ERK1/2, JNK, and p38) and the AKT cell survival pathway (AKT, PTEN, and glycogen synthase kinase 3β) after PEP005 treatment in Colo205-sensitive and HT29-resistant cell lines. PEP005 activated Raf by dephosphorylation at Ser^{259} (Fig. 3A). The dephosphorylation of Ser^{259} is required in order for activation of Raf1 downstream signaling (31). As shown in Fig. 3A, PEP005 induced a rapid dephosphorylation of Ser^{259}, detected at 5 min. The activation of Raf was associated with the increased phosphorylation of ERK1/2, JNK, and p38 MAPK in Colo205-sensitive cell line (Fig. 3A). Therefore, treatment of cells with PEP005 resulted in an activation of the MAPK pathway.

PEP005 also down-regulated AKT1 activity (Fig. 3B). After 10 min of treatment, we observed a significant decrease (~10-fold) of AKT1 phosphorylation without any detectable changes in AKT1 protein level. Multiple studies of phosphatidylinositol metabolism indicate that PTEN plays a critical role as a negative regulator of phosphatidylinositol 3-kinase (PI3K) and AKT signaling. In our study, we also showed a rapid activation of PTEN in response to PEP005 (Fig. 3B).

PP2A is the protein phosphatase that targets phospho-AKT, rendering it inactive. In our study, AKT inhibition by PEP005 was reversed by pretreatment with okadaic acid, a specific inhibitor of PP1 and PP2A phosphatases (Fig. 3C). PEP005 thus inhibits a well-documented cell survival pathway, AKT, and this may contribute to its apoptosis-inducing effects.

To determine which signaling pathways lay downstream or were activated in parallel to PKC, the consequences of inhibiting PKC for PEP005-induced effects on MAPKs and AKT were determined. Colo205 cells were pretreated with rottlerin (PKCδ inhibitor) and chelerythrine chloride (pan-PKC inhibitor) followed by PEP005 addition for 5 to 60 min (Fig. 4A). Chelerythrine pretreatment gave a partial inhibition of the effects of PEP005 on ERK1/2 and p38 MAPK phosphorylation but did not affect the inhibition of AKT phosphorylation by PEP005, suggesting that PEP005 affects the MAPK via activation of PKC. Moreover, we showed that pretreatment of cells with rottlerin blocked activation of ERK1/2 and also had a modest effect on p38 activation by PEP005 (Fig. 4A). Rottlerin also had no effect on AKT dephosphorylation induced by PEP005. Therefore, PKCδ seems to mediate PEP005 effects on ERK1/2 signaling and to a lesser extent on p38 signaling.

To study the biological consequences of inhibiting MAPKs and JNK, Colo205 cells were pretreated for 2 h with UO126 (MEK1/2 inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor) followed by PEP005 addition for 5 to 60 min (Fig. 4A). Inhibition of MEK1/2 by UO126 resulted in an almost complete inhibition of ERK1/2 phosphorylation and a dramatic decrease of its regulation by PEP005. p38 kinase was not phosphorylated by PEP005 after UO126 pretreatment, indicating close cross-talk between the MAPK and p38 signaling pathways. SB203580 pretreatment inhibited p38 activity and diminished the basal level of AKT phosphorylation. Jun kinase inhibition by SP600125 also led to AKT dephosphorylation and inhibited the PEP005-dependent p38 phosphorylation.

To determine the effects of inhibiting PI3K, which lies upstream of AKT, and MAPKs on PEP005-induced apoptosis, Colo205 cells were pretreated for 2 h with wortmannin (PI3K inhibitor) or UO126 and the cells were then treated with PEP005 for 30 and 60 min and apoptosis was determined by measurement of active caspase-3 by Western blot. Using both pharmacologic inhibitors, we

found inhibition of PEP005-induced caspase-3 activation (Fig. 4B). Taken together, these data support the hypothesis that PEP005-induced apoptosis is mediated through activation of MAPK and inhibition of PI3K/AKT pathways, with the former lying downstream of activation of PKCδ.

Signaling Events in PEP005-Resistant Cells

In contrast to Colo205, HT29 cancer cells were shown to be resistant to PEP005. Modulation of the various signaling events shown above to be involved in PEP005-induced apoptosis was therefore examined in HT29 cells. Figure 5 shows that PEP005 induced only minimal and transient activation of PKCδ, detected as the appearance of phospho-PKCδ. There was limited only and transient activation of p38 and MAPK, and in addition, PEP005 had no effect on the phosphorylated status of AKT and PTEN (Fig. 5). Phosphorylation of PKCα was down-regulated after PEP005 treatment in HT29 cells, as was observed in Colo205 cells, but there was no initial activation of this PKC.

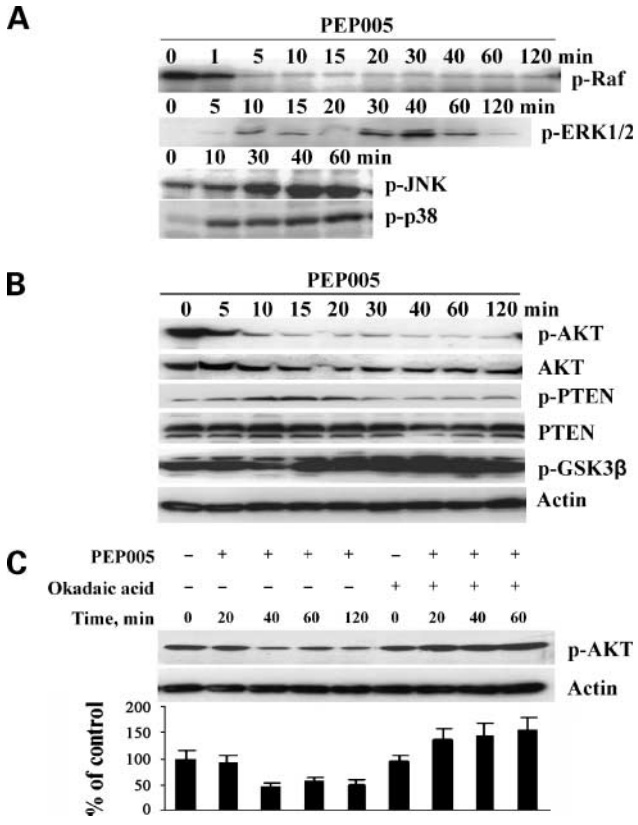


Figure 3. Kinase activation by PEP005 in Colo205 cells. Colo205 cells were treated by 0.3 μmol/L PEP005 for the indicated times and analyzed for phospho-Raf (*p-Raf*), phospho-ERK1/2 (*p-ERK1/2*), phospho-JNK (*p-JNK*), and phospho-p38 (*p-p38*) expression (**A**) and phospho-AKT (*p-AKT*) and total AKT, phospho-PTEN (*p-PTEN*) and total PTEN, and phospho-glycogen synthase kinase 3β (*p-GSK3β*; **B**). The blots shown are representative of three separate experiments done. **C,** Colo205 cells were pretreated with okadaic acid (10 nmol/L) for 2 h and then treated with PEP005 and tested for phospho-AKT expression. *Bottom,* densitometric analysis of Western blot.

Discussion

In this study, antiproliferative effects and apoptosis induction by PEP005 were observed over a broad concentrations ranging from 0.001 to 300 μmol/L in cells derived from various solid tumors. Interestingly, we observed that some cell lines (Colo205 and MDA-MB-435) were exquisitely sensitive to PEP005 with IC_{50} <3 μmol/L, contrasting with limited antiproliferative effects in most of the other cell lines examined. Because the optimal antiproliferative effect was observed with prolonged exposure of cancer cells, our results suggest that protracted or repeated dosing may be required to maximize pharmacologic effects of PEP005 in clinical trials. Furthermore, our data suggest that cancer cell death induced by PEP005 is not dependent on tumor type but rather on characteristics related to specific activation of signaling pathways by PEP005 in cancer cells.

Because PEP005 is thought to act through the modulation of PKC isoforms (24), we investigated the PKC expression in our panel of cell lines. PEP005-sensitive Colo205 cells displayed a high level of expression of several PKC isoforms, notably PKCα and PKCδ, which show the highest affinity for PEP005 *in vitro* (24). However, other cell lines that were resistant to PEP005 also expressed several PKC isoforms, suggesting that altered PKC expression did not explain sensitivity to PEP005 in the cell lines tested here. Our data showing that high levels of PKCδ expression are associated with sensitivity to PEP005 are consistent with that previously reported in myeloid leukemia (21).

To further understand the basis of PEP005-induced apoptosis, we examined activation of PKC isoforms and also other signaling pathways known to regulate either cell proliferation or cell survival. PEP005 induced a transient increase in phospho-PKCδ in Colo205 cells, which was followed by a gradual loss of this isoform in the phosphorylated and nonphosphorylated forms. The activation of PKCδ was further confirmed by its translocation to the nucleus and other cellular membranes. This observation is consistent with that reported in leukemic cells (21). Nuclear translocation has been shown previously to be associated with the proapoptotic functions of PKCδ and in particular its role as an apoptotic lamin kinase (4). Activation of PKCδ was shown to induce cellular death in several models, including keratinocytes (32), salivary gland acinar cells (33), vascular smooth muscle cells (5), leukoblasts (21, 34), and colon cancer cells (35). In addition, the eventual loss of PKCα may also play a role in the balance between cellular proliferation and apoptosis, as this isoform is antiapoptotic in several human tumor models (2, 8). Interestingly, it was expressed at very high level in many of the cell lines tested and was in an active phosphorylated state in Colo205 cells. Consequently, cellular effects of PEP005 on PKCs in Colo205 cells may be seen as a gain of function, appropriately restoring the balance between survival and death in cancer cells.

Treatment of cells with PEP005 also resulted in the modulation of multiple signaling pathways, including p38 MAPK, ERK1/2, and JNK. Our data also suggest that activation of ERK1/2 lies downstream of PKCδ and that

activation of both PKCδ and ERK1/2 is required for induction of apoptosis by PEP005. It may seem counter-intuitive that molecules promoting cell survival, such as MAPK, may be up-regulated by PEP005, although the drug may still be capable of inducing antiproliferative effects and apoptosis. It was previously shown that active ERKs phosphorylate numerous cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors, and cytoskeleton proteins, inducing growth, proliferation, differentiation, and migration, but in some circumstances may also induce apoptosis (reviewed in ref. 36). Activation of the Ras/MEK/ERK pathway induces the stabilization of c-Myc and its proapoptotic function, which may in part account for proapoptotic effects. Results similar to our data were obtained by Cozzi et al. (37) in melanoma cells. Considering our results, it is tempting to suggest that the antiproliferative effects of PEP005 may be driven by inhibition of AKT that is acknowledged to play a major role in cell survival. Recent studies (37) have reported that ERK1/2 was phosphorylated in response to PEP005 treatment in melanoma cell lines regardless of their sensitivity to diterpene ester treatment. Conversely, our data suggest that sensitivity may be dependent on the activation of MAPK signaling pathway, as HT29 cells were resistant to PEP005 and ERK1/2 was also not activated following treatment with PEP005. Interestingly, pharmacologic inhibition of PKC using a broad-range inhibitor chelerythrine resulted in complete blockade of the activation of p38, whereas inhibition of PKCδ with rottlerin gave only a partial inhibition of p38 activation by PEP005 but a complete block in apoptosis. These data suggest that p38 MAPK is activated by PKC

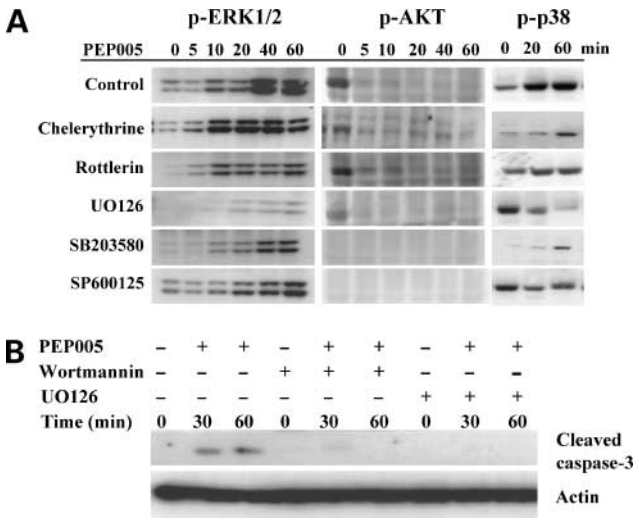


Figure 4. **A,** effects of PKC, MAPK, and JNK inhibitors on PEP005 signaling. Colo205 cells were pretreated with chelerythrine (5 μmol/L), rottlerin (5 μmol/L), UO126 (10 μmol/L), SB203580 (20 μmol/L), and SP600125 (20 μmol/L) for 2 h, treated with 0.3 μmol/L PEP005, and evaluated for phospho-ERK1/2, phospho-AKT, and phospho-p38 expression. **B,** inhibition of PI3K and MEK1/2 blocks PEP005-mediated apoptosis. Colo205 cells were pretreated with wortmannin and UO126 for 2 h and then treated with 0.3 μmol/L PEP005; protein extracts were analyzed for cleaved activated fragment of caspase-3.

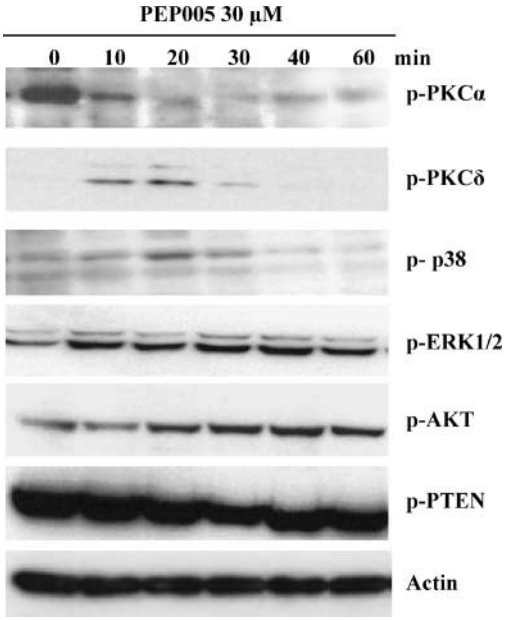


Figure 5. Kinase activation by PEP005 in HT29 cells. HT29 cells were treated with 30 μmol/L PEP005 and probed for detection of phospho-PKCα, PKCδ, p38, ERK1/2, AKT, and PTEN by Western blotting. The blots shown are representative of three separate experiments done.

isoforms in addition to PKCδ but also that it does not seem to play a major role in PEP005-induced cell death in Colo205 cells. Further studies may attempt to determine which of the molecular targets PKCα or PKCδ account the most in apoptosis induced by PEP005 in cancer cells. Other reports have shown the role of p38 in PKCδ-induced apoptosis via accumulation and phosphorylation of p53 (5). Although depletion of p53 in Colo205 would have been ideal to show the role of p53 in PEP005 sensitivity, our data suggest that p53 does not play a major role in PEP005-induced apoptosis; this further supports a lack of involvement of p38 in PEP005-induced apoptosis in Colo205 cells. In addition, modulation of most of the signaling pathways that was seen in Colo205 cells treated with PEP005, including PKC, p38 MAPK, AKT, and PTEN, did not occur in HT29 cells. This would suggest that these resistant cells were able to prevent initial access of PEP005 to these proximal signaling pathways. Whether PEP005 could be sequestered or removed from these cells, possibly via the actions of drug efflux pumps, remains to be determined.

Colo205 cells express high levels of phospho-AKT, a kinase that has been implicated in mediating cell survival in many cell types. For example, inhibition of AKT function leads to apoptosis in prostate cells (38), as the PI3K/AKT pathway has a central role in protecting prostate cancer cells from tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis. We have shown that PEP005 treatment leads to a rapid dephosphorylation of AKT in sensitive Colo205 cells associated with concomitant activation of PTEN, a lipid phosphatase that acts as a negative regulator of the PI3K/AKT pathway. Thus, in Colo205 cells, PEP005 treatment results in the inhibition of survival

signaling. However, this modulation of AKT was not abrogated by chelerythrine or rottlerin and it is therefore possible that PEP005 is able to regulate either PI3K/AKT or PTEN directly or via a PKC-independent intermediate. In this regard, the AKT dephosphorylation depends on PP2A phosphatase activity (39). Because treatment with okadaic acid blocked the PEP005 effect on AKT, it is possible that PP2A is activated by PEP005. This clearly requires further study as it may confirm a non-PKC target for PEP005.

In summary, our data provide novel insights into the complexity of the signaling pathways controlled by PKC isozymes and the mechanisms regulating cell survival in colon cancer cells. Our results strongly suggest that in Colo205 colon cancer cells both AKT and Ras/Raf/MAPK pathways are differentially modulated by PEP005 and that only the latter is mediated by PKC isozymes. Tumor cells that are insensitive to PEP005 showed a lack of activation of PKC and MAPK and no down-regulation of AKT, and this may allow identification of tumors that may preferentially benefit from PEP005 in clinical trials.

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